



# THE JOURNAL OF BIOLOGICAL CHEMISTRY

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MEMORIAL FUND

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VOLUME 170  
BALTIMORE  
1947

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THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC

PUBLISHED AT YALE UNIVERSITY FOR  
THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC  
WAVERLY PRESS, INC  
BALTIMORE 2, U S A

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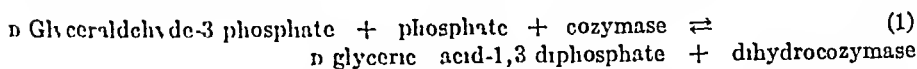
# THE MECHANISM OF THE OXIDATIVE REACTION IN FERMENTATION\*

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(Received for publication, April 8, 1947)

The reaction discovered by Warburg and Christian (1) has been the



subject of a previous study by Drabkin and Meyerhof (2). One of the problems to be settled was the form in which glyceraldehyde phosphate took up inorganic phosphate prior to oxidation. Some data were obtained which suggested the formation of an addition compound. However, the results were not as satisfactory as might be desired, since it was not possible with the spectrophotometric equipment used by Drabkin and Meyerhof (2, 3) to follow the course of the reaction (development of the absorption band at  $340 m\mu$  of dihydrocozymase) from the moment of mixing with the enzyme. This, together with the relatively high corrections for the blank, caused some uncertainty as to whether the final equilibrium was established. The present investigation, made with a Beckman spectrophotometer, did not have these disadvantages.

Since the publication of our first paper, Cori and coworkers (4) have described the crystallization of an oxidative enzyme from muscle extract which has properties similar to those of the enzyme from yeast. Cori's enzyme requires the presence of glutathione (or cysteine) in relatively high concentration during the course of the reaction. Glutathione and cysteine react with triose and methylglyoxal, producing substances which absorb at  $340 m\mu$ . Since this complicates the measurement, we used Cori's enzyme only to establish the fact that the equilibria are identical with those obtained with Warburg's enzyme from yeast. This confirms the conclusion drawn from other observations that the final values obtained represent true thermodynamic equilibria. Indeed, it will be shown below that the requirements of the law of mass action are excellently fulfilled for every component of the system at equilibrium. On the other hand, there is only a slight indication of the formation of an addition product of glyceraldehyde phosphate and phosphate.

\* This work was aided by grants from the Penrose Fund of the American Philosophical Society and the Dazian Foundation for Medical Research.

or, if [diphosphoglyceric] is replaced by  $h$  as before, and  $g'$  and  $p'$  kept constant, equation (11) becomes

$$r = \frac{K^*}{H^+} \quad (12)$$

or in logarithmic form

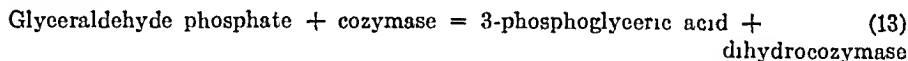
$$\log r = \text{pH} + \log K^* \quad (12, a)$$

Over the range from pH 8.3 to 5.5, this equation holds. This is the greatest possible range, since in a more alkaline solution cozymase and triose phosphate decompose and in a more acid medium dihydrocozymase decomposes. Three series of experiments gave the following results (cf. Fig. 4)

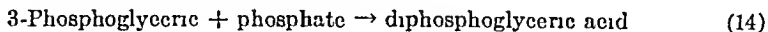
pH changed from	8.28	to	6.31	or	-1.97,	log $r$ changed	-2.10
"	"	"	8.27	"	5.42	"	-2.85, " " " -2.92
"	"	"	8.0	"	5.58	"	-2.42, " " " -2.52

We assume, therefore, that equation (12) gives the true equation of the thermodynamic equilibrium.<sup>2</sup>

It may be added that change of temperature has no influence on equilibrium (2). At 22° and 32° exactly the same  $r$  values are found for identical mixtures. If  $dK/dT \sim 0$ , the reaction must be thermonutral according to van't Hoff's reaction isochore. Since it was formerly found (7) that the reaction



gave about 7500 to 10,000 calories per mole, a similar amount of heat must be consumed in the endothermic reaction



## II Methods and Preparations

Most of the methods used here have already been described in former papers (2, 7). However, the Beckman spectrophotometer enabled us to

<sup>2</sup> Since with  $g'$ ,  $p'$ , and pH constant  $r = K'''$  (equation (9)), equation (12, a) leads to the conclusion that the system

$$\frac{[\text{diphosphoglyceric}]}{[\text{glyceraldehyde phosphate}] \times [\text{phosphate}]}$$

should give an oxidation reduction potential with a 0.058 volt shift per unit of pH and should resemble the system  $[\text{pyruvate}^-]/[\text{lactate}^-]$  since no ions are created or destroyed (see foot-note 1). Per unit increase of pH,  $\Delta \log K = 1$ ,  $-RT \log K = -1340$  calories, or  $\Delta E/\text{pH} = RT/2F = -0.029$  volt. But the system  $\text{DPN}^+ + 2e + \text{H}^+ \rightleftharpoons \text{DPNH}$  itself shows a 0.029 volt shift per pH unit (see Clark (6)). Therefore the total equilibrium follows the equation  $+0.029 \text{ volt}_{K'''} = x - 0.029 \text{ volt}_{\text{DPN}}$ , where  $x$  is  $-\Delta E/\Delta \text{pH}$  for the diphosphoglyceric system.  $x$  is therefore = 0.058 volt

determine the absorption of the background, which depends mostly on the amount of triose phosphate. When this had been done, 0.1 to 0.2 cc of the diluted enzyme was added. Readings were taken every minute until they became constant. When the background was not quite stable, a cell filled with all components except the protein served as a control. In all later experiments we used the cooling block through which water at room temperature circulated.

The oxidizing enzyme was prepared according to Warburg and Christian, with the modifications already described (7). The enzyme could be preserved for several weeks with 0.025 M glutathione. Since the enzyme is diluted 100 to 200 times during the measurement, the final concentration of glutathione is less than  $2 \times 10^{-4}$  M, and has practically no influence on the background absorption. Cozymase was purified from a sample kindly supplied by Hoffmann-La Roche.<sup>3</sup> The preparation was made according to Williamson and Green (8) from baker's yeast. By this procedure a purity of 55 to 60 per cent is attained with fewer steps and with a higher yield than by the method of Ohlmeyer (9, 10) used in our former work. But further purification is much more difficult. The final purity was about 65 or 70 per cent after correcting for the 7.7 per cent of water present, while the P content was 8.7 per cent after drying (theory 9.35 per cent). We measured the purity from the maximum value of absorption at 340 m $\mu$  obtained in the presence of the oxidizing enzyme and arsenate, deducting the absorption of the background, and using for  $\epsilon$  ( $d = 1$  cm,  $c = 1$  mM per liter) 6.27, as determined by Ohlmeyer.<sup>4</sup>

Triose phosphate, hexose diphosphate, aldolase, and isomerase were prepared and purified as described formerly (7). For purposes of comparison we also used the racemic glyceraldehyde phosphate of Baer and Fischer (11).<sup>5</sup> Calculated on the basis of alkali-saponifiable P, this product gave exactly the same value for  $h$  as half the amount of D-glyceraldehyde phosphate of our "natural" product. This confirmed the correctness of our calculations of  $g'$ .

The glyceraldehyde used was a crystallized preparation from Schering-Kahlbaum.

The pH was determined with a Leeds and Northrup pH meter and glass electrode. Sodium pyrophosphate or veronal-acetate according to Michaelis (12) usually served as buffer. But we also tested sodium triphosphate, which behaved like pyrophosphate, and alanylglycine, which gave values identical with those of veronal-acetate at pH 8.

<sup>3</sup> We thank Dr. Aeschlimann of Hoffmann-La Roche, Inc., for supplying us with 5 gm of this cozymase.

<sup>4</sup> Calculated from Ohlmeyer's coefficient,  $\epsilon = 0.4343 \times \beta 10^{-4}$ .

<sup>5</sup> We thank Dr. Fischer and Dr. Baer for kindly supplying us with two samples of their preparation.

To ascertain the maximum amount of orthophosphate which the solution of pyrophosphate contained, a modification of the Fiske-Subbarow method was employed. Each of a series of Nessler tubes is filled with 13 to 9 cc of distilled water, 1 to 5 cc of the unknown, 5 cc of ethyl alcohol (95 per cent), and 1 cc of eikonogen, they are cooled to about  $10^{\circ}$ , the molybdate- $\text{H}_2\text{SO}_4$  reagent is added last, and the contents of the tubes are mixed immediately. The full color develops within a few seconds on account of the accelerating effect of alcohol. If, with this procedure, solutions of orthophosphate containing 3 to 10  $\gamma$  of P per tube are compared with solutions of pyrophosphate or with mixtures of both, it can be estimated from the color in the first seconds after mixing whether the pyrophosphate contains less than 3  $\gamma$  of P of orthophosphate or more and how much. With this method we found that sodium pyrophosphate (Mallinckrodt, analytical reagent) does not contain a measurable amount of orthophosphate (less than 3  $\gamma$  of P per 1 cc of 0.04 M solution), but if neutralized to pH 8.2 and kept some days, it contains around 3  $\gamma$  of P, and if neutralized to pH 7 it contains much more, and so on, the more acid it is made. The lowest concentrations of  $p'$  on the curves (Fig. 1) in the presence of pyrophosphate are corrected with the values determined in this way.

### III Dependence of $r$ on $p'$ in Presence of Various Buffers

With either veronal-acetate, alanyl-glycine, or pyrophosphate as buffer the curves for  $\log r$  versus  $\log p'$  for nearly constant  $g'$  are straight lines, as shown in Figs. 1 and 2, for pH 8.3 and 7.15. (Since the added amount of glyceraldehyde phosphate was somewhat over  $10 \times 10^{-4}$  mole per cc, while  $h$  was between  $1 \times 10^{-4}$  and  $0.1 \times 10^{-4}$ ,  $g'$  in these experiments did not change more than 10 per cent. This change if applied as a correction would not amount to more than 0.04 on the logarithmic curve for a variation of  $\log p'$  of 2 to 3, or to less than 2 per cent or  $1^{\circ}$  of slope.) Three different slopes are visible in Figs. 1 and 2, corresponding to the three cases: (1) indifferent buffer, (2) pyrophosphate buffer, (3) no buffer. That veronal-acetate and alanyl-glycine can be termed indifferent buffers follows not only from the fact that the straight lines usually have the theoretically expected slope of  $45^{\circ}$  but that for the same pH the curves coincide. In the presence of pyrophosphate the line has a slope of a little less than  $45^{\circ}$ , which seems to show that pyrophosphate enhances the effect of phosphate. On the other hand, in the absence of buffer the line is much steeper and somewhat curved towards the abscissa. This latter course is undoubtedly connected with the lowering of the ionic or osmotic strength with decreasing phosphate concentrations, because other salts like  $\text{Na}_2\text{SO}_4$  or KCl also increase the values of  $r$  for low phosphate concentrations and raise the curve similarly to a straight line of  $45^{\circ}$ .

The occasional deviations from the 45° slope of indifferent buffer are probably due to small changes of pH in such cases, in which the pH of the veronal-acetate, the orthophosphate, and the other components was not exactly the same. Indeed, with the variation of the phosphate concentration over a range from  $500 \times 10^{-3}$  to  $0.5 \times 10^{-3}$  such concomitant changes are unavoidable (Fig 1). The same result can be seen from the constancy of  $K'$  in Table I. The amount of buffer is also an important factor. 0.03 mole gives about the maximum effect. Lower concentrations give inter-

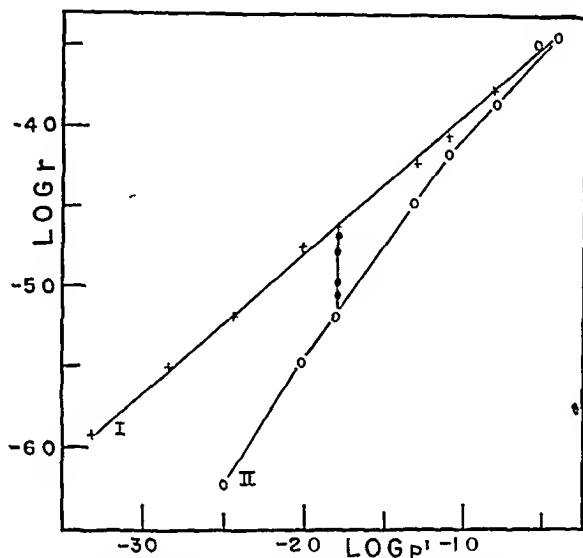


Fig 1 The relationship at equilibrium of  $\log r$  to  $\log p'$  with approximately constant  $g'$  ( $r = [\text{dihydrocozymase}]^2/[\text{cozymase}]$ ,  $p'$  inorganic phosphate,  $g'$  glyceraldehyde phosphate at equilibrium), Experiment 5.7 pH 7.15. Curve I buffered with  $29 \times 10^{-3}$  M pyrophosphate, Curve II no buffer added. The vertical line at  $\log p' = -1.8$  gives  $r$  values with various concentrations of pyrophosphate (cf Table II, Experiment 5.9).

mediate values between that obtained with 0.03 M buffer and without. This is shown for  $15 \times 10^{-3}$  M phosphate in Fig 1 where points on the vertical line represent  $r$  values obtained with different amounts of pyrophosphate. These points are taken from Table II.

A word may be added about the effect of very high concentrations of  $p'$ . At pH 8.3 the equilibrium of equation (1) is so far to the right that even with 0.08 M phosphate 95 per cent of the added cozymase may be oxidized. In this case an error of 1 per cent in the total cozymase gives more than 20 per cent variation in  $r$ . Such points are necessarily inaccurate. On the other hand, at pH 7 with 0.08 M phosphate only 50 to 60 per cent of the



cozymase is reduced. The values of  $r$  can be determined very accurately and can be followed for a phosphate concentration much higher still. Under these conditions the line bends to a somewhat flatter slope and  $0.57 \text{ M } p'$ , which is 33 per cent greater than  $0.43 \text{ M}$ , gives a value of  $r$  only 20 per cent greater. However, this may be attributable to the same influence of ionic concentration as the steepening of the curve at low salt concentration.

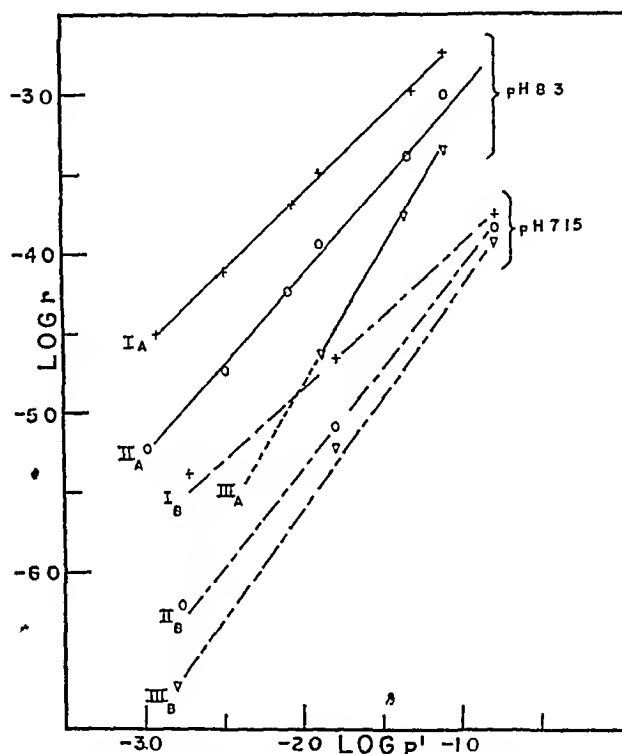


FIG. 2.  $\log r$  plotted against  $\log p'$  with constant  $g'$  at pH 8.3 (A) and 7.15 (B), Experiment 5-15. Curves I, pyrophosphate as buffer ( $29 \times 10^{-3} \text{ M}$ ), Curves II, veronal-acetate ( $30 \times 10^{-3}$ ) and alanylglycine ( $40 \times 10^{-3}$ ), giving virtually the same points, Curves III, no buffer.

Another explanation would be the formation of a small amount of an addition product, which would diminish the value of  $g'$  about 25 per cent in the presence of  $150 \times 10^{-3} \text{ M}$  phosphate and somewhat more in the presence of  $500 \times 10^{-3} \text{ M}$  phosphate (cf. Section VII).

In Table I, Experiments 5-1 and 5-2, Cori's crystallized oxidizing enzyme from muscle was used. The measurements were duplicated at the same time with the yeast enzyme and gave identical values (not shown in

TABLE I  
Variation of  $r$  with  $p'$  at Constant  $g^*$

Experiment No	pH	Buffer	$h \times 10^4$	$r \times 10^4$	$p' \times 10^3$	$g' \times 10^3$	$K = \frac{r}{g' \times p'}$
4-4	8.2	Pyrophosphate	0.897†				
			0.870	28.0	71.7	1.03	(37.7)‡
			0.840	12.4	43.0	1.035	27.8
			0.746	3.68	14.5	1.045	24.3
			0.670	1.97	8.8	1.05	21.3
			0.536	0.78	3.09	1.065	23.6
			0.408	0.343	1.11	1.08	28.6
			0.227	0.077	0.27	1.10	25.9
5-1 Corn enzyme	8.2	"	0.873				
			0.818	12.1	78.0	1.15	12.8
			0.765	5.4	46.5	1.16	10.0
			0.607	1.39	15.5	1.17	7.7
			0.527	0.805	9.55	1.18	7.2
			0.398	0.333	3.36	1.19	8.3
			0.271	0.122	1.15	1.21	8.8
			0.145	0.029	0.23	1.22	10.0
5-7	7.15	Orthophosphate only	1.580				
			1.195	3.74	370	1.01	1.00
			1.170	3.35	309	1.01	1.07
			0.960	1.49	154	1.03	0.94
	7.15	Pyrophosphate	0.994	1.69	154	1.03	1.06
			0.817	0.878	77	1.05	1.09
			0.717	0.598	46.5	1.06	1.21
			0.508	0.241	15.6	1.08	1.42
			0.457	0.190	9.44	1.085	1.84
			0.298	0.0695	3.48	1.10	1.76
			0.209	0.0320	1.40	1.11	2.05
			0.134	0.0124	0.47	1.12	2.35
5-2 Corn enzyme	7.15	"	0.880				
			0.492	0.623	78	1.42	0.56
			0.437	0.43	46.5	1.43	0.65
			0.311	0.17	15.5	1.44	0.76
			0.249	0.098	9.58	1.45	0.71
			0.168	0.040	3.34	1.45	0.80
			0.110	0.0157	1.17	1.46	0.92
			0.062	0.0047	0.235	1.465	(1.37)‡
1-15	6.95	Orthophosphate only	1.560				
			1.164	3.42	549	0.99	0.63
			1.112	2.76	412	1.00	0.67
			1.040	2.08	275	1.01	0.75
			0.848	1.01	137	1.03	0.72
			0.708	0.583	82.4	1.04	0.68

TABLE I—*Concluded*

Experiment No	pH	Buffer	$h \times 10^4$	$r \times 10^4$	$p' \times 10^3$	$g' \times 10^3$	$K = \frac{r}{g' \times p'}$
4-8	6.0	Pyrophosphate	0.895 0.191 0.120 0.089 0.062 0.035	0.0517 0.0186 0.0098 0.0046 0.0014	71.7 28.7 14.5 5.95 1.67	1.10 1.11 1.11 1.11 1.12	0.066 0.058 0.061 0.070 0.075

\* For the symbols used see the explanation in Section I of the text

† The bold-faced value is the maximum value determined in the presence of  $3 \times 10^{-3}$  M arsenate and equals the total cozymase present

‡ Inaccurate values

TABLE II

*Salt Effect*

Experiment 5-9 pH 7.15, total cozymase =  $1.59 \times 10^{-4}$ ,  $p' = 15.7 \times 10^{-3}$ ,  $g' = 1.0 \times 10^{-3}$

Salt added	Concentration $\times 10^3$	$h \times 10^4$	$r \times 10^4$
Pyrophosphate	0	0.288	0.064
	2.85	0.334	0.0895
	5.7	0.366	0.111
	11.4	0.428	0.160
	28.5	0.496	0.228
K <sub>2</sub> SO <sub>4</sub>	142	0.374	0.115
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	115	0.327	0.085
KCl	110	0.378	0.119

Table I) Repetition of Experiment 1-15 at 32° instead of 22° gave the same values

*IV Dependence of r on g' at Constant Phosphate Concentration*

The straight logarithmic lines obtained are shown in Fig 3. Because the concentration of  $p$  is about 100 times as great as that of  $h$  in *maximo*, no corrections for the bound phosphate in diphosphoglyceric acid need be applied. In this case background absorption has to be determined for all concentrations of  $g'$ .

Since no additional complications arise here by the variation of the small amounts of triose phosphate, as in the case of the variation of phosphate, the logarithmic lines show an exact slope of 45°. Some further experiments are collected in Table III.

*V Dependence of r on pH and Independence of Total Cozymase*

The points of Fig 4 were obtained in two experiments in which the pH was varied. The straight line is drawn at an angle of 45°. The true curve

is a little concave to the abscissa, but this deviation is close to the margin of experimental error. The constancy of  $r$  with variation of the amount of added cozymase is shown in Table IV, Experiment 12-23. The variation of  $p'$  plays no rôle on account of the high concentration of  $p$ , but  $g'$  decreases with increasing  $h$  from  $0.93 \times 10^{-3}$  to  $0.855 \times 10^{-3}$  M. This corresponds to a variation of 8 per cent in the  $r$  value. In the last two columns the actual values are compared with the "theoretical" value of

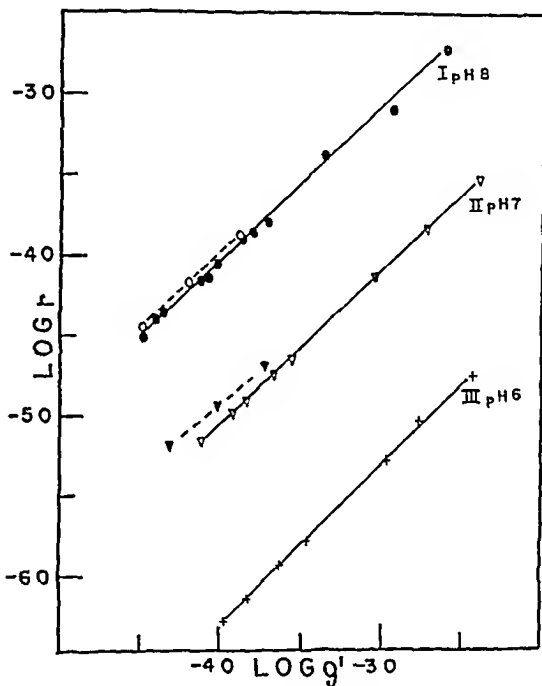


FIG. 3.  $\log r$  plotted against  $\log g'$  with constant  $p'$ , Experiments 7-9 and 4-13. Curve I, pH 8, Curve II, pH 7, Curve III, pH 6.0. The dotted lines indicate  $r$  values determined in the presence of isomerase (see Section VI).

$r$ , assuming that the value of  $r = 0.62 \times 10^{-4}$  for  $1.06 \times 10^{-4}$  mole of added cozymase is exact. Although the remaining unreduced cozymase varies 100-fold, the experimental value of  $r$  agrees with the theoretical values to within  $\pm 15$  per cent, well inside the experimental error.

#### VI. Equilibrium in Presence of Oxidizing Enzyme and Isomerase

The experiments described above have only a slight bearing on the question of an addition compound of phosphate and glyceraldehyde phosphate. If  $p'$  is kept high and practically constant, the concentration  $a$  of the addition product would be proportional to  $g'$  and the straight line would not differ from that actually obtained. If  $p'$  is varied over a large range, a

difference in the curve should become visible if  $a$  is a large fraction of  $g'$ . In this case equation (4) would be transformed into

$$\log r = \log (g - h - a) + \log (p - h - a) + \log K \quad (15)$$

TABLE III  
*Variation of  $r$  with  $g'$  at Constant  $p'$*

Experiment No	pH	Buffer	$h \times 10^4$	$r \times 10^4$	$p \times 10^3$	$g' \times 10^3$	$K$
4-12	8.2	Pyrophosphate	1.600				
			1.546	44.3	71.5	3.43	18.1
			1.489	19.9	71.5	1.64	17.0
			1.450	14.0	71.5	0.93	21.0
			1.300	6.64	71.5	0.381	24.8
			1.205	3.67	71.5	0.248	20.7
			1.095	2.38	71.5	0.146	22.9
			1.010	1.73	71.5	0.104	23.3
			0.900	1.16	71.5	0.062	26.2
			0.518	0.263	71.5	0.0107	34.3
7-9	7.0	Veronal-acetate	1.635				
			1.170	2.94	156	3.78	0.50
			0.990	1.52	156	1.85	0.53
			0.795	0.75	156	0.895	0.54
			0.590	0.332	156	0.373	0.57
			0.512	0.233	156	0.273	0.55
			0.476	0.196	156	0.223	0.56
			0.395	0.125	156	0.149	0.54
			0.370	0.108	156	0.125	0.55
			0.302	0.069	156	0.078	0.57
4-13	6.0	Pyrophosphate	1.280				
			0.402	0.184	71.5	3.54	0.073
			0.290	0.095	71.5	1.76	0.076
			0.233	0.0515	71.5	1.05	0.069
			0.163	0.0238	71.5	0.495	0.067
			0.139	0.0169	71.5	0.344	0.069
			0.120	0.0124	71.5	0.242	0.072
			0.093	0.0073	71.5	0.142	0.072

$g'$  would become proportionally greater at lower phosphate concentration, the slope would be less than  $45^\circ$ , and the log curve would become concave towards the abscissa in the higher range of phosphate concentrations.

But a better and more direct way of demonstrating the presence and the amount, if there is any, of such an addition product consists in combining the equilibrium of equation (2) with a second enzymatic equilibrium. This has been discussed in the foregoing paper. If we add isomerase to triose phosphate, equilibrium (16) is established,

$$K_{\text{isom}} = \frac{g'}{d_i} = 0.042 \quad (16)$$

where  $g'$  is the equilibrium concentration of glyceraldehyde phosphate and  $d_i$  the concentration of dihydroxyacetone phosphate. When the total added triose phosphate is  $t$  and no other reaction occurs,

$$t = g' + d_i \quad (17)$$

In the presence of cozymase and oxidizing enzyme, however, part of the glyceraldehyde phosphate is turned into diphosphoglyceric acid, equal to

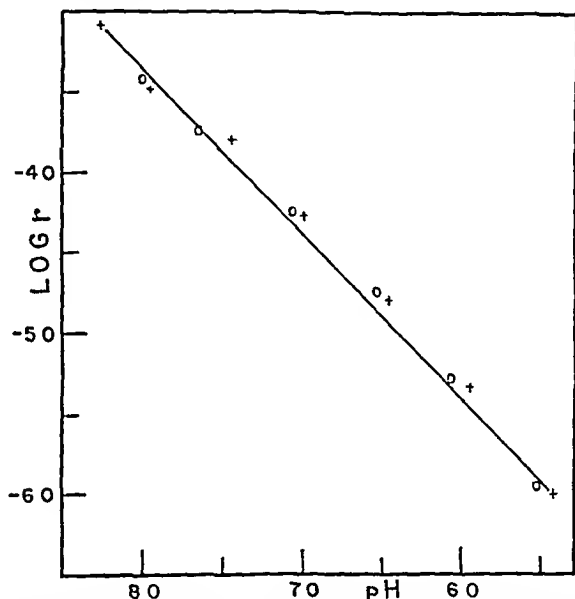


FIG 4  $\text{Log } r$  plotted against pH.  $1.6 \times 10^{-4} \text{ M}$  cozymase,  $150 \times 10^{-3} \text{ M}$  phosphate  $0.85 \times 10^{-3} \text{ M}$  glyceraldehyde phosphate. X, Experiment 12-19, O Experiment 12-23

$h$  In this case in the presence as well as in the absence of isomerase

$$t = g' + h + d_i \quad (17, a)$$

In the absence of isomerase  $d_i$  and  $g'$  are equal to the directly determined contents of the two esters, while in its presence they have to be calculated from equation (16)

If part of the added triose ester formed an addition product  $a$ , equation (17, b) would hold,

$$t = g' + h + a + d_i \quad (17, b)$$

We, therefore, determine first for various amounts of added triose phos-

phate the values of  $h$  with and without addition of isomerase. This is followed up by a second series of experiments with such small additions of triose phosphate that the results with isomerase are exactly matched in regard to  $h$ . Since in the absence of isomerase,  $g - h = g' + a$ , we can, after plotting  $\log r$  versus  $\log (g - h)$ , read off from the  $r$  values of the first series the values of  $g' + a$  in the presence of isomerase. We can then determine from equation (17, b) the value of  $dr$  and see whether  $(g' + a)/dr$  is larger than  $g'/dr = K_{\text{isom}} = 0.042$ . Of course, this will be the case if  $a > 0$ . We shall call  $(g' + a)/dr$  the "apparent  $K_{\text{isom}}$ ".

TABLE IV

*Variation of  $r$  with Variation of Cozymase*

For glyceraldehyde phosphate,  $p' = 18 \times 10^{-3}$ , D-glyceraldehyde (Experiment 2),  $g' = 14.5 \times 10^{-3}$ . All in veronal-acetate, pH 8

Experiment No	Total cozymase $\times 10^4$	$h \times 10^4$	$a \times 10^4$	$g' \times 10^3$	$r$ found $\times 10^4$	$r$ calculated* $\times 10^4$
12-23 Glyceraldehyde phosphate	2.12	0.844	1.276	0.865	0.534	0.60
	1.06	0.558	0.502	0.885	0.620	0.62
	0.530	0.340	0.190	0.905	0.608	0.63
	0.265	0.196	0.069	0.920	0.558	0.64
	0.106	0.094	0.012	0.930	0.737	0.65
				$p' \times 10^3$		
12-7 D-Glyceraldehyde	2.07	1.14	0.93	0.74	1.40	1.40
	1.035	0.696	0.340	0.78	1.42	1.47
	0.518	0.384	0.134	0.81	1.10	1.53
	0.259	0.230	0.029	0.83	1.82	1.57

\* Calculated on the assumption that one of the found values is correct, and that the others may then be determined from the variation in  $g'$  or  $p'$ .  $K = r/(g' \times p')$

There is undoubtedly some tendency to obtain values of the apparent  $K_{\text{isom}}$  higher than 0.042. The average of twenty-one different measurements made under various conditions is 0.051. The individual values are scattered fairly widely between 0.039 and 0.076. But the results become clearer when the following experimental findings are added. In the average the excess over the theoretical value of  $K_{\text{isom}}$  is greater when  $p'$  is  $144 \times 10^{-3}$  M than when it is  $43 \times 10^{-3}$  or  $72 \times 10^{-3}$ . In the eight experiments with  $144 \times 10^{-3}$   $p'$ , the values are between 0.045 and 0.076 and the average is 0.057. Moreover the values are generally higher for pH 7 than for pH 8, and for the lowest range of added triose phosphate, i.e. under conditions in which  $r$  is relatively small. Then the value for the apparent  $K_{\text{isom}}$  lies around 0.06. The fact that  $a$  is greatest when  $r$  is smallest is confirmed by the results obtained with zymohease (see Section VII). By the combination of the two enzymes, isomerase and aldolase, the concentration of

$g'$  comes into the lower brackets, and the nine best experiments of that series give an average of 0.059 for the apparent  $K_{\text{isom}}$

The results obtained with isomerase are reproduced in Table V. This enzyme was purified as described previously by Meyerhof and Beck (13), but only to the stage of fractional precipitation with ammonium sulfate. Since it is only stable in the presence of dilute ammonium sulfate (about 2 per

TABLE V  
*Equilibrium of Isomerase and Oxidizing Enzyme*

Experiment No	pH	$p \times 10^3$	Total triose phosphate ( $i-h$ ) $\times 10^3$	$r \times 10^3$	$(g-h) \times 10^3$ *	Apparent† $K_{\text{isom}}$
3-21	8.2	43	2.4	0.345	0.093	0.039
3-22	8.2	43	4.53	0.864	0.251	0.055
3-26	7.0	72	4.53	0.116	0.226	0.045
			2.40	0.0572	0.108	0.0405
			1.20	0.0440	0.0785	0.059
4-12	8.0	72	4.90	3.46	0.232	0.0475
			2.41	1.68	0.100	0.0415
			1.42	1.07	0.057	0.040
			0.644	0.526	0.028	0.043
4-12	7.0	72	4.98	0.185	0.255	0.051
			2.47	0.103	0.135	0.055
			1.48	0.065	0.086	0.058
4-13	6.0	72	2.50	0.0053	0.104	0.042
7-8	8.0	144	3.00	1.28	0.136	0.045
			1.51	0.688	0.072	0.048
			0.73	0.347	0.34	0.046
7-9	7.0	144	4.60	0.220	0.255	0.056
			2.28	0.117	0.138	0.061
			1.13	0.064	0.072	0.063
10-4	8.2	144	0.270	0.160	0.0166	0.061
10-14	8.0	144	0.275	0.129	0.021	0.076

\*  $g-h$  determined from  $r$

†  $(g' + a)/d_i$ , see Section VI of the text

cent), this has to be added. It was more advantageous to add the oxidizing enzyme first and thus establish the equilibrium with the high concentration of glyceraldehyde phosphate in about 1 minute or less, and then add the isomerase, which causes the value of  $h$  to diminish in the course of a few minutes to a new steady value. If the sequence is reversed, this same equilibrium-value is reached more slowly, probably because the reaction of the oxidizing enzyme starts in this case with a very low concentration of glyceraldehyde phosphate.

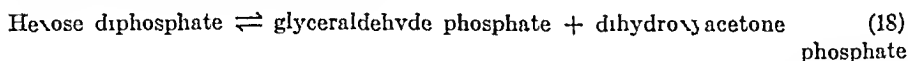
The activity of the isomerase used was always determined first by the



procedure already described (7) Glyceraldehyde phosphate was oxidized by iodine and the rotation of D-3-phosphoglyceric acid determined in the presence of molybdate Such amounts of isomerase were added to the complete oxidizing system as caused 95 per cent of the equilibrium amount of dihydroxyacetone phosphate to be formed in 2 to 4 minutes at room temperature About 10 to 15 minutes were allowed in the spectrophotometric measurement In Table V only those experiments satisfactory in all these respects are reproduced

#### - VII *Equilibrium in Presence of Oxidizing Enzyme and Zymohexase*

The same problem can be broached with the system hexose diphosphate (or triose phosphate) + zymohexase (aldolase + isomerase) in addition to the oxidizing enzyme and the other components As was reported previously (2) in some instances values for the addition product of the order of half of  $g'$  ( $a \cong 0.5 g'$ ) were obtained Such an arrangement, in which three distinct enzymes are needed to establish the equilibrium, is more liable to errors than the former one, containing only two enzymes Evidence as to whether the final equilibrium is established can only be gained by following closely the time course of the absorption until it becomes steady This could not be done with the older arrangement, but was easy with our present equipment If to hexose diphosphate and the complete oxidizing system a purified preparation of zymohexase is added, which may contain an excess of aldolase over isomerase, the course of the absorption goes through a maximum This is due to the circumstance that at first the equilibrium of aldolase prevails



The amount of glyceraldehyde phosphate diminishes subsequently as the isomerase equilibrium is established The minimum value after the hump of the curve is then taken as the final equilibrium of zymohexase This end-value was obtained more easily when the oxidizing enzyme was put in first and zymohexase added later This must be explained, as in the case of isomerase, by the low concentration of glyceraldehyde phosphate with which the reaction starts when the zymohexase equilibrium is already established

These experiments are not so easily evaluated as those with isomerase alone To find the total triose phosphate in the equilibrium we formerly determined the alkali-labile P by incubating a similar system with the same amount of zymohexase, but with inorganic phosphate replaced by another buffer of the same pH We found now that relatively high concentrations of phosphate diminish the amount of triose phosphate in the equilibrium in the same way as do other salts (14) With  $75 \times 10^{-3} M$  phosphate the

decrease in the equilibrium value of dihydroxyacetone phosphate was about 10 per cent, with  $150 \times 10^{-3}$  M about 25 per cent of the value found with veronal buffer. This was determined by the Ariyama method for methylglyoxal, which we used after 60 minutes of hydrolysis in N HCl at  $100^\circ$ , as described in former papers (15). Therefore, in the final experiments we replaced the determination of alkali-labile P by the determination of methylglyoxal after hydrolysis. This determination is not influenced by the phosphate present and can be carried out in the same samples which

TABLE VI

*Equilibrium of Zymohexase and Oxidizing Enzyme*

Veronal acetate buffer, pH 8

Experiment No	$p \times 10^3$	Added phosphate ester	Total organic P at equilibrium $\times 10^3$	Triose* phosphate at equilibrium $\times 10^3$	$r \times 10^4$	$(g-h) \times 10^3$	Apparent $K_{isom}$
5-27	156	Hexose	9.0	2.4	0.72	0.082	0.038
	156	"	9.0	2.4	0.88	0.100	
5-29	156	"	6.1	1.9	0.89	0.101	0.053
6-11	156	"	7.7	2.8	1.05	0.172	0.061
	156	Triose	8.0	2.9	1.05	0.172	0.060
7-25	156	Hexose	7.7	2.13	1.13	0.151	0.064
	156	"	7.7	2.13	0.98	0.126	
	156	Triose	7.7	2.15	0.97	0.125	0.056
	156	"	7.7	2.15	0.85	0.116	
11-8	156	"	0.93	0.72	0.33	0.052	0.070
	156	"	0.93	0.72	0.30	0.049	
11-15	75	"	5.6	Not determined	0.263	0.133	0.069
	75	Hexose	4.9	1.46	0.177	0.091	0.063

\* Determined by methylglyoxal analysis or from alkali labile phosphate in a separate sample after incubation with zymohexase in absence of inorganic phosphate

have served for the absorption measurement and which have been deproteinized with trichloroacetic acid. Methylglyoxal present before hydrolysis must be determined in every sample and subtracted. With suitable reduction of the amounts of the reagents the Ariyama method gives reliable results with 5  $\gamma$  of methylglyoxal per 10 cc upwards. Only a part of the experiments could be utilized for the final calculation. In five cases starting from the side of hexose diphosphate and four from the side of triose phosphate, the absorption experiments were made in duplicate or triplicate, and either the amounts of added enzymes or the sequence of additions varied. When nearly the same value for  $r$  was obtained, the mean was used for calculation. When the measurements varied but seemed equally reliable, the  $(g-h)$  values are enumerated separately in Table VI.

But since only one determination of methylglyoxal (or alkali-labile P) served for the final calculation, they are combined in the evaluation of the apparent  $K_{180m}$ . These values were scattered from 0.038 to 0.070 and gave an average of 0.059. Since all except the last two experiments were made in the presence of  $145 \times 10^{-3}$  M phosphate, they should be compared with the eight isomerase experiments with this same concentration, for which the mean was 0.057 for apparent  $K_{180m}$ . There seems to be no

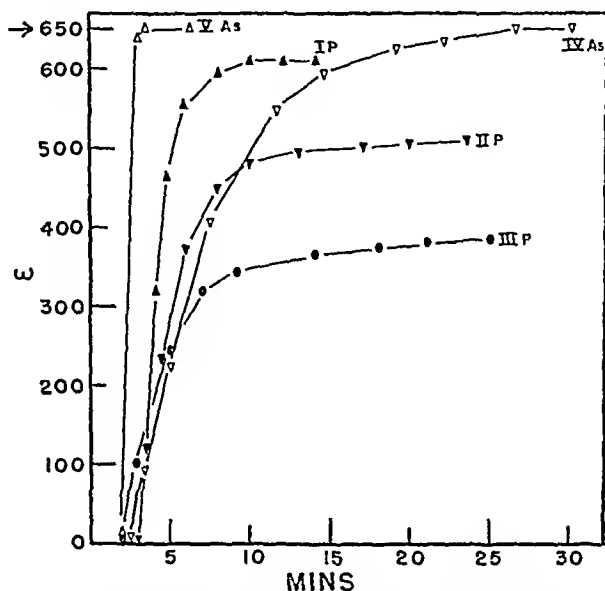


FIG 5 Time course of  $\epsilon$  at  $340\text{ m}\mu$  in the presence of free glyceraldehyde, 0.4 cc of undiluted enzyme at pH 8 (3.0 mg),  $1.03 \times 10^{-4}$  M cozymase (3.5 cc). Curve I,  $1.8 \times 10^{-2}$  M phosphate, Curve II,  $1.8 \times 10^{-3}$  M, Curve III,  $4.2 \times 10^{-4}$  M, Curve IV,  $2.4 \times 10^{-3}$  M arsenate, for comparison Curve V, triosephosphate and  $2.4 \times 10^{-3}$  M arsenate, with 0.4 cc enzyme diluted twelve times. For Curve V the maximum absorption is obtained in less than 1 minute, in Curve IV after 25 minutes but the end-values are the same.

doubt that in this way the presence of an addition product can be demonstrated, although in very small amount, equal to one-fourth to one-fifth of  $g'$ .

#### VIII Oxidation of Free Glyceraldehyde by Cozymase

Warburg and Christian have established the fact that free glyceraldehyde also reacts with cozymase in the presence of phosphate if about 1000 times as much enzyme is used. We can confirm this result, we needed about 100 to 200 times as much enzyme as in the former case for establishment of a stable equilibrium. Fig 5 shows a curve with arsenate (Curve IV),

which allows the oxidation of the total amount of cozymase as in the presence of glyceraldehyde phosphate, and some curves with inorganic phosphate (Curves I, II, III) At pH 8  $\log r$  plotted against  $\log p'$  gives a straight line, proving in principle the same relationship as with the ester. But this straight line is flatter than  $45^\circ$  both with veronal buffer and with pyrophosphate buffer. Indeed both give practically identical results (Fig. 6).

At pH 8,  $30 \times 10^{-3}$  M phosphate gives more than 95 per cent reduction of cozymase, and higher concentrations of phosphate which apparently cause 100 per cent reduction cannot be used for evaluating the logarithmic curves.

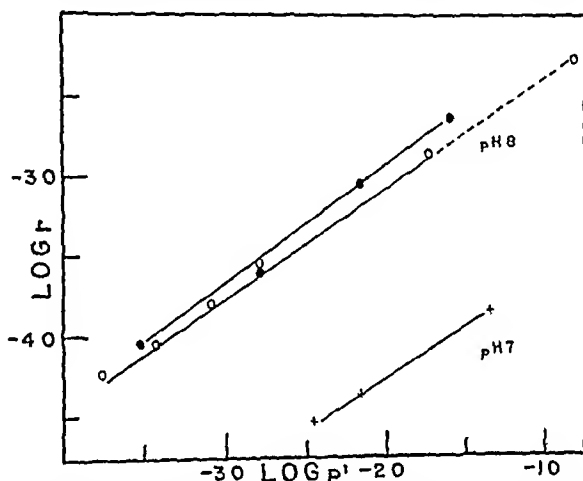


FIG. 6.  $\log r$  plotted against  $\log p'$  with a constant amount of D glyceraldehyde,  $7 \times 10^{-3}$  M. O, Experiment 12-11 with veronal-acetate, pH 8, ●, Experiment 1-7 with pyrophosphate, pH 8, X, Experiment 12-13 with veronal acetate, pH 7. Only the points with medium  $p'$  concentrations are reproduced (Higher concentrations inhibit).

That the curves are straight lines with a flatter slope means that for lower phosphate concentrations the reduction is relatively greater than with the glyceraldehyde ester. In repeating the measurements at pH 7 we found that the affinity of the cozymase for moderate concentrations of phosphate is lowered in the same way as was found in Section IV for glyceraldehyde phosphate. But by increasing the phosphate concentration above  $30 \times 10^{-3}$  M, the value for  $r$  drops again, the more so the higher the phosphate concentration. Probably the reactivity of the enzyme is impaired. That in general the reaction with glyceraldehyde follows nearly the same pattern as with its ester does not seem doubtful. This can best be demonstrated by changing the amount of total cozymase while  $p'$  and glyceraldehyde remain constant. Here equation (9),  $r = K'''$ , holds as in the other case. This is shown in Table IV, Experiment 12-7, in which the added

cozymase is changed over a range of 8 l, oxidized cozymase changes 30 l, and  $\gamma$  is constant within the accuracy of measurement ( $\pm 28$  per cent). The equilibrium here can be formulated in the same way as equation (2). But since the affinity of the enzyme is so much less, as is proved by the large amounts necessary, secondary influences apparently complicate the picture more than in the case of glyceraldehyde phosphate.

#### DISCUSSION

The oxidative reaction of fermentation obeys equation (2) so quantitatively that the formation of any large amount of a dissociable diphosphoglyceric aldehyde is precluded. If such a compound formed,  $\log p'$  plotted against  $\log \gamma$  with constant buffer concentration would not give a straight line, as it does in Fig. 1, Curve I, but a curve bent concavely towards the abscissa. The curvature would be greater the larger the relative amount of the compound, or the smaller the dissociation constant.

$$K_{diss} = \frac{p' \times g'}{a} \quad (19)$$

It is questionable whether the slight bending of the log curve for higher concentrations than 0.15 M phosphate should be ascribed to such a combination. Even if it should, this would mean that only above such a concentration would an appreciable amount of the compound be formed. The upper limit is clearly given by the results with isomerase and zymohecase, which show that in the presence of 0.15 M phosphate not more than about a quarter of the 0.001 mole of glyceraldehyde phosphate present exists in this form.

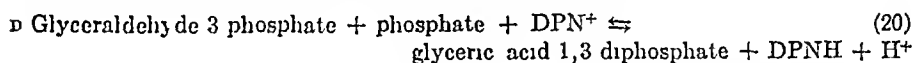
If 25 per cent existed at this phosphate concentration in bound form and equation (19) is applied, then at  $3 \times 10^{-3}$  M the compound  $a$  would equal 0.5 per cent. Nevertheless, with such an amount of phosphate at pH 8 about half of the cozymase present ( $1.5 \times 10^{-4}$  M) will still be reduced at equilibrium. This shows that such equilibrium considerations cannot explain the reactivity of glyceraldehyde phosphate in the presence of phosphate in our system. It is indeed probable that this loose addition takes place only in the presence of the oxidizing enzyme and cozymase and is not detectable outside of their field of action. This argument was recently used by Lipmann (5) for the analogous case of oxidation of pyruvic acid in the presence of phosphate to acetyl phosphate. In both instances the combination with phosphate is very much looser than with sulfite. With 0.1 M sulfite an appreciable change in the absorption of the aldehyde group of glyceraldehyde phosphate at 265  $m\mu$  can be observed, but no certain change can be observed in the presence of 0.15 M phosphate.

One may even consider the slight increase in the total ester in the combined oxidation and isomerization equilibria to be due not so much to the

formation of an addition product as to a stabilizing effect of the isomerization on the oxidizing reaction. But such an interpretation is not very probable. Of all the components of the reaction, diphosphoglyceric acid is the most unstable. Its decomposition would increase  $k$  rather than decrease it. We, therefore, prefer the explanation that the average slight increase in the combined equilibrium over that which is calculated is an indication of the addition, but only in the sphere of the oxidizing enzyme.

The zymohexase and isomerase equilibria in themselves show, therefore, no increase of total esterified glyceraldehyde in the presence of phosphate, a negative result formerly obtained with Junowicz-Kocholaty (7). One argument of this paper, however, should be revised. It was assumed that if such a dissociation equilibrium as equation (19) existed independently of the oxidative step,  $K_{dis}$  could not be larger than 0.01 to 0.05, since a greater constant was incompatible with the allegedly maximum effect of a concentration of 0.03 mole of phosphate on the reduction of cozymase. This maximum does not exist, the value of  $r$  increases appreciably even above 0.3 M phosphate at a suitable pH. This argument, therefore, is fallacious. From our values of the apparent  $K_{isom}$  such a dissociation could have  $K$  0.5 or higher.

From the relationship of  $\log r$  to pH it follows that the equilibrium reaction of oxidation should be formulated as follows:



The requirements of the law of mass action for this equilibrium are shown to be fulfilled for every component of the system in the whole range investigated, which extends for the different components from 1/25 to 1/1000. This is even true for the diphosphoglyceric acid, which was not tested directly but taken as equimolecular to dihydrocozymase.

#### SUMMARY

1. The mechanism of the oxidative reaction of fermentation was investigated by means of the absorption of dihydrocozymase at 340 m $\mu$ , measured with a Beckman spectrophotometer. Under suitable conditions the reaction obeys the law of a thermodynamic equilibrium in regard to all participants. This is shown for D-glyceraldehyde phosphate, inorganic phosphate, cozymase, and H<sup>+</sup> ion. The apparent deviation which was found previously (2) in respect to inorganic phosphate is due to a secondary influence, the change of ionic strength, and can be eliminated by addition of enough indifferent buffer. The dependence of the equilibrium on the concentration of cozymase and on C<sub>H</sub><sup>+</sup> is an additional proof for the validity of the equation formulated.

2. If the equilibrium of the oxidative reaction is coupled with the other

enzymatic equilibria of isomerase and aldolase, it can be shown, as originally found by Drabkin and Meyerhof (2), that the established concentration of glyceraldehyde phosphate is slightly higher than the equilibrium of isomerase requires. This small increase in the mean concentration, amounting to about 25 per cent, is taken as an indication that an unstable addition product forms, but apparently only in the presence of the oxidizing enzyme and cozymase.

3 Free glyceraldehyde behaves similarly to the triose ester. Moreover, it reacts with arsenate in the same way as does the latter.

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# THE IN VITRO METABOLISM OF TESTOSTERONE TO $\Delta^4$ -ANDROSTENEDIONE-3,17, *cis*-TESTOSTERONE, AND OTHER STEROIDS BY RABBIT LIVER SLICES\*

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(Received for publication, February 10, 1947)

The administration of testosterone, an androgen present in the testes of bulls (1, 2) and horses (3), or its propionate to hypogonadal men and various animals causes an increased excretion of the known urinary androgens (1). The amount and nature of the material excreted apparently vary with the species. The rat (4) and dog (5) excrete only small amounts of biologically active androgens, and man (1) relatively large amounts. Furthermore, the apparent<sup>1</sup> chief substances excreted by man are androsterone and its ring isomer, etiocholanol-3( $\alpha$ )-one-17, while that of the guinea pig is isoandrosterone (1). The administration of androstenedione-3,17,  $\Delta^4$ -androstenedione-3,17, androstenediol-3( $\alpha$ ),17( $\alpha$ ), (1,7), and dehydroisoandrosterone (8) also cause an increased excretion of the known urinary androgens. On the basis of the above evidence it has been postulated that the urinary androgens are end-products of the metabolism of testosterone and that  $\Delta^4$ -androstenedione-3,17, androstenedione, and androstenediol-3( $\alpha$ ),17( $\alpha$ ) may be intermediates in this process. A similar scheme has been postulated for the metabolism of dehydroisoandrosterone (8) to androsterone. It is recognized, however, that these data provide only indirect evidence for such a scheme. Therefore, in order to obtain more direct information concerning the sites, modes, and products of metabolism of the steroid hormones, *in vitro* studies on the effect of various tissues on the different steroids have been initiated. This report is concerned with the effect of liver slices from adult male rabbits on testosterone.

\* This investigation was supported by grants (to C. D. K.) from the Committee for Research in Endocrinology of the National Research Council.

The authors are indebted to Miss Julia Lobotsky for assistance, especially with the chromatographic analyses and the colorimetric determinations.

† The data in this paper were taken from a thesis presented by L. C. Clark, Jr., to the Graduate School of The University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy, October, 1944. Present address, Samuel S. Fels Institute for Research in Human Development, Antioch College, Yellow Springs, Ohio.

<sup>1</sup> Unfortunately, all of these studies have been directed toward the isolation of the known androgens in urine. Exhaustive explorations of the urine, especially the hydroxy non-ketonic fraction, may reveal other compounds (cf. (6)).



### Procedure

The testosterone was suspended by homogenization in a 1:1 serum-Ringer-phosphate buffer mixture at 1 mg per ml and incubated with rabbit liver slices for  $2\frac{1}{2}$  or 3 hours. At the end of the incubation period 10 volumes of 95 per cent ethyl alcohol were added, and the precipitated protein was removed by filtration and exhaustively extracted with organic solvents. The extracts were pooled, concentrated to a sludge, and extracted with benzene and ether. The phospholipides were removed and the residue divided into ketonic and non-ketonic materials by means of Girard's Reagent T. These fractions were separated further by fractional

TABLE I  
*In Vitro Metabolism of Testosterone by Rabbit Liver Slices*

Compound		Control		Experiment 1		Experiment 2	
		mg	per cent*	mg	per cent*	mg	per cent*
Material added	Testosterone	1000		2000		1000	
" isolated†	Ketonic steroids	1093	109.3	1720	86.0	901	90.1
	Testosterone	937	93.7	1026	51.3	511	51.1
	cis-Testosterone			12	0.6	12	1.2
	$\Delta^4$ -Androstenedione-3,17			98	4.9	85	8.5
	$C_{19}H_{28}O_3$ (m p 201-203°)					4	0.4
	Naturally occurring compounds‡						
	Total cholesterol	244		536		520	
	Hydrocarbon (?) (m p 60°)	+		7		+	
	Carotenoid (?) (m p 193.5°)	3		3		+	

\* Per cent of added material

† No evidence for the presence of androsterone and related steroids

‡ No attempt was made to obtain quantitative recoveries. In some instances, indicated by +, the substances were recognized by their chemical behavior

crystallization, chromatographic adsorption, and chemical reagents. Two experiments and a control experiment without the incubation period were run in this manner. The amounts of testosterone used for each experiment were 2.00, 1.00, and 1.00 gm respectively.

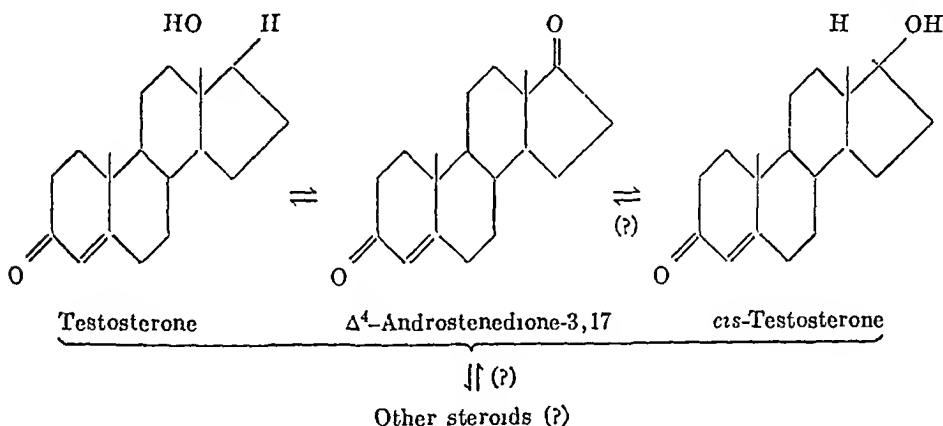
### Results

The crude ketonic material obtained in the control experiment was 9.3 per cent greater,<sup>2</sup> while the same fraction of the incubation experiments was 14 and 10 per cent less than the added testosterone (Table I). The

- Similar recoveries have been obtained in several experiments with cell-free liver preparations which did not metabolize testosterone (unpublished)

loss of material could not be accounted for by the products isolated from the non-ketonic fraction or by the formation of acid-hydrolyzable conjugated material. A further loss of materials was experienced in isolating the pure substances from the ketonic fraction.

$\Delta^4$ -Androstenedione-3,17 and *cis*-testosterone were the only two metabolites present in sufficient amounts to be isolated and characterized. The greatest recovery was obtained in Experiment 2, in which 8.5 and 1.2 per cent of the added testosterone was recovered as the respective metabolites. Undoubtedly, much more of these materials was formed than isolated since considerable loss may be expected in the necessary manipulative procedures. This fact explains the lower yields in Experiment 1, in which more manipulations were necessary. The products isolated, however, suggest that the 17( $\alpha$ )-hydroxyl group is readily oxidized to a ketone group, which in turn is reduced either only to the 17( $\beta$ )-hydroxyl group,



or to both the 17( $\beta$ )- and 17( $\alpha$ )-hydroxyl groups (see the accompanying structures). If the latter is the case, then some of the isolated testosterone has gone through a process of oxidation and reduction back to its original form.<sup>3</sup>

It is of particular significance that *cis*-testosterone has not been obtained from natural sources and  $\Delta^4$ -androstenedione-3,17 has been isolated only from the adrenal cortex, probably as an artifact (9). Several other substances were obtained in too small amounts to characterize completely. A partial characterization, however, was accomplished by the use of various color tests (Zimmermann as modified by Holtorff and Koch (10), Pincus (11), and Kagi-Miescher 17-hydroxyl and 17( $\beta$ )-hydroxyl (12)), digitonin precipitation (Reichstein (13)), and in one instance by carbon and hydrogen

<sup>3</sup>  $\Delta^4$ -Androstenedione 3,17 is converted to testosterone and *cis*-testosterone by incubation with rabbit liver slices (unpublished).

analyses<sup>4</sup> One of these compounds was of special interest because it had the composition  $C_{19}H_{28-30}O_3$  The amount of material was not sufficient to permit further characterization

The presence of only trace amounts of 3-hydroxy compounds suggests that this position in the testosterone molecule either is not attacked readily or else the action is so severe that the products are degraded to hitherto unrecognized or non-steroid compounds, *e g* acids The latter would provide some explanation of the inability to account for all of the added testosterone

Since androsterone and its isomers are apparently the chief androgens excreted in the urine of man after the administration of testosterone (1), an exhaustive search was made for these compounds and also the phenolic estrogens, but without any success This, however, does not disprove the hypothesis that testosterone may be a precursor of these urinary steroids, because not only may it be differently metabolized in the rabbit, but also other tissues, *e g* the kidney (*cf* (14)), may be involved The formation of the isolated metabolites by bacterial action (*cf* (1)) has been ruled out by the use of sterile conditions in Experiment 2

In the course of the isolation procedure several other well known naturally occurring compounds were obtained (Table I) No attempt was made to characterize or isolate these in quantitative amounts

#### EXPERIMENTAL

*Melting Points*—All of the melting points were determined with Anschütz thermometers on an electrically heated aluminum block<sup>5</sup> placed under a microscope Calibration of the apparatus with standard substances indicated that true values were obtained as read

*Testosterone*—The material<sup>6</sup> contained a trace of yellow impurity which was removed by recrystallization from carbon tetrachloride The clean white needles, after drying over phosphorus pentoxide, melted at 154.0–154.5° and on analysis gave carbon and hydrogen values corresponding to those of testosterone As a further check on the purity of the testosterone, 150 mg of the recrystallized material were submitted to succinoylation The trace amount of oily non-hydroxy residue gave negative reactions in the Zimmermann (10), Pincus (11), and Kagi-Miescher (12) color tests

*Incubation Procedure*—Non-fasted, adult male rabbits of mixed breeds

<sup>4</sup> The carbon and hydrogen microanalyses were performed by Dr Donald Ketchem, Research Laboratories, Eastman Kodak Company

<sup>5</sup> The details of the construction of this apparatus will be described elsewhere

<sup>6</sup> The testosterone and other steroids used in this study were generously provided by Ciba Pharmaceutical Products, Inc, through the courtesy of Dr E Oppenheimer and Dr C R Scholz

were killed by bleeding through the jugular vein. The blood was defibrinated and the serum separated. 10 ml of the serum were homogenized with 100 mg of testosterone in an all-glass apparatus of the Potter-Elvehjem type (15). The resulting suspension was diluted with 40 ml of serum, then with 50 ml of Ringer-phosphate buffer, pH 7.4, and poured into a Fernbach flask which was loosely stoppered with non-absorbent cotton. In Experiment 1, 200 to 300 mg of tissue slices and 3 ml of the substrate mixture were incubated in each of a series of 50 ml Erlenmeyer flasks. The Fernbach flask proved to be more satisfactory for large scale incubation.

While the blood was being centrifuged, the liver was removed, sliced into sections 0.20 to 0.25 mm thick, and collected in chilled Ringer-phosphate buffer. Approximately 10 gm of these slices were transferred to the Fernbach flask containing the substrate mixture. The flask was shaken at 100 revolutions per minute for 3 hours in Experiment 1 and  $2\frac{1}{2}$  hours in Experiment 2 in a water bath maintained at 37.0°. The incubation was terminated by adding 10 volumes of redistilled ethanol to the contents of the flask.

*Extraction and Gross Fractionation*—After the alcohol incubation mixture had stood at room temperature between 20 and 24 hours, the protein precipitate was removed by filtration and washed three times with boiling 95 per cent ethanol. The moist precipitate was mixed with an equal volume of purified Hyflo Super-Cel<sup>7</sup> (a diatomaceous earth) and extracted into a Soxhlet apparatus for two 8 hour periods with ethanol, one 8 hour period with acetone, and one 8 hour period with benzene. The Soxhlet extracts and the ethanol filtrate were concentrated *in vacuo* (water pump) at 35° to one-fortieth of the original volume. This aqueous concentrate was thoroughly extracted with ether and benzene, the extracts were combined and distilled in a partial vacuum to give the "total fat-soluble" fraction. Further extraction of the aqueous fraction with ethyl acetate yielded only a very small additional amount of dark oil from which nothing could be isolated.

The phospholipides were precipitated from an ether solution of the total fat-soluble material by the addition of dry acetone and a few drops of a saturated solution of magnesium chloride in ethanol. The acetone-soluble fraction yielded a brown-orange oil which was separated into ketonic and non-ketonic substances with Girard's Reagent T (16). These gross fractions, ketonic and non-ketonic, were further separated and will be considered in detail for each experiment.

<sup>7</sup> The Hyflo Super-Cel and Celite were generously provided by Johns-Manville. They were extracted with ethanol for 8 hours in a Soxhlet apparatus and dried in an electric oven at 100° and then in a muffle furnace at 500°.

### *Control Experiment*

In this series of ten experiments the liver slices were placed in the Fernbach flasks, and the substrate mixture and then the ethanol were immediately added

*Recovery of Testosterone*—Repeated recrystallization of the ketonic fraction from methanol-water gave 0.846 gm of a crystalline white product, which melted at 153–154°. Mixed melting points with testosterone showed no depression. Carbon and hydrogen analyses agreed with the theoretical values for testosterone.

The mother liquors from the crystallizations were combined and concentrated to give a yellow oil, 0.133 gm. This oil was dissolved in anhydrous carbon tetrachloride and adsorbed on 9.0 gm of magnesium silicate-Celite<sup>7, 8</sup> in a column 25 mm × 50 mm. The eluates yielded 91 mg of testosterone, m.p. 149–152°. Thus, the total amount of crystalline testosterone recovered from the ketonic fraction was 0.937 gm or 93.7 per cent of the amount added to the substrate mixture.

*Fractionation of Non-Ketonic Material*—The non-ketonic residue, 1.874 gm, was hydrolyzed with 10 per cent potassium hydroxide in methanol. The saponifiable material was recovered, but was not studied further.

The unsaponifiable residue (0.519 gm) was dissolved in anhydrous carbon tetrachloride, adsorbed on 20 gm of magnesium silicate-Celite in a column 40 mm wide and 60 mm long, and eluted progressively with carbon tetrachloride, benzene, ether, methanol, and then respective mixtures until twenty-eight fractions were obtained. The absence of 17-hydroxy steroids in all of the fractions was indicated by color tests (10, 11). Three fractions (Nos. 7 to 9) consisted of 244 mg of cholesterol, m.p. 146–147°. Carbon and hydrogen analyses agreed with the theoretical values. The acetate melted at 112–114°.

A small amount, about 3 mg, of an orange-colored crystalline material melting at 193.5° was recovered from the last two (strongly adsorbed) fractions. The substance gave a blue-violet color when dissolved in concentrated sulfuric acid. The nature of this compound is not known. Similar amounts of apparently identical material were found in the experiments reported below.

### *Incubation of Testosterone, Experiment 1*

A total of 2 gm of testosterone was incubated in twenty-two experiments with liver slices from twenty-two rabbits of various strains. No special asepsis except through cleanliness was employed.

<sup>8</sup> Dr. K. Dobriner, Memorial Hospital, New York, kindly provided his unpublished method for the use of this adsorbent.

Bioassay by the rat method used in this laboratory (17) indicated the absence of androgenic activity in the acetone-insoluble fraction and a 20 per cent loss in total activity in the acetone-soluble fraction

The ketonic material was fractionally crystallized from methanol-water into several crystalline fractions, which were pooled into two portions in accordance to their melting points, these are designated as Fraction A and Fraction B and the mother liquors as Fraction C

*Recovery of Testosterone*—Fraction A was repeatedly crystallized from dilute methanol to give 0.902 gm of white needles, m p 151–152° A mixed melting point with an authentic sample of testosterone showed no depression The oxime melted at 221–225° and showed no depression when mixed with an authentic sample of testosterone oxime, m p 221–222° The benzoate melted at 192–193° and did not depress the melting point of testosterone benzoate, m p 193–194° A negative response to both the Zimmermann and the Pincus color tests indicated the absence of 17-keto steroids

*Isolation of  $\Delta^4$ -Androstenedione-3,17*—Fraction B was a white powder, 147 mg, and m p 130–138° It gave a strong violet color with the Zimmermann reaction, indicating the presence of a 17-keto steroid, which was not androstosterone or its stereoisomers because it gave a negative Pincus reaction A 60 mg aliquot of this material was separated into non-hydroxy ketonic and hydroxy ketonic fractions by succinylation

Recrystallization of the non-hydroxy ketonic fraction from ether-pentane gave 34 mg of needles which melted at 166–168.5°, and when mixed with an authentic sample of  $\Delta^4$ -androstenedione-3,17, m p 169–170°, the mixture melted at 167–169.5° The dioxime melted at 139–142° and gave no depression in melting point when mixed with an authentic sample of  $\Delta^4$ -androstenedione-3,17 dioxime, m p 141–143° The 2,4-dinitrophenylhydrazone had a deep red-orange color characteristic of  $\alpha,\beta$ -unsaturated ketonic steroids Since 34 mg of  $\Delta^4$ -androstenedione-3,17 were isolated from a 60 mg aliquot, a total of 83.4 mg was calculated to be present in Fraction B

Hydrolysis of the succinates of the hydroxy ketones by refluxing for 2 hours with methanolic 10 per cent potassium hydroxide yielded 25 mg of free steroids as a yellow oil Separation into  $\alpha$ - and  $\beta$ -steroids with digitonin gave 5 mg of  $\beta$ -hydroxy ketones and 19 mg of  $\alpha$ -hydroxy ketones Attempts to isolate pure compounds from these materials were unsuccessful

*Isolation of cis-Testosterone, Additional Testosterone, and  $\Delta^4$ -Androstenedione-3,17*—The mother liquors from the crystallization of the ketonic material were concentrated *in vacuo* to give 177 mg of an oil from which crystalline material could not be obtained by high vacuum sublimation This oil, therefore, was dissolved in 10 ml of carbon tetrachloride, adsorbed

on a column of aluminum oxide (Blockmann) 0.5 cm  $\times$  30 cm, and selectively eluted with increasing amounts of ethanol in carbon tetrachloride (18) (Table II). A total of 14 mg of  $\Delta^4$ -androstenedione-3,17, 39 mg of testosterone, and 12.5 mg of *cis*-testosterone was isolated. The *cis*-testosterone upon recrystallization melted at 218–221° and did not depress the melting point of an authentic sample of *cis*-testosterone. It gave a brown color in the Zimmermann reaction, which corresponded in intensity (photoelectric colorimeter) to that given by a sample of pure *cis*-testosterone. Application of the Kagi-Miescher test for the 17( $\beta$ )-hydroxyl group gave a deep violet color with a strong red fluorescence.

*Fractionation of Non-Ketonic Hydroxy Fraction*—This fraction, 0.650 gm was subjected to fractional crystallization, molecular distillation, and chromatographic analysis. A total of 0.446 gm of cholesterol, m.p. 145–146.5°, was isolated and identified by its acetate, m.p. 112–114°, and

TABLE II  
*Chromatographic Analysis on Al<sub>2</sub>O<sub>3</sub> of Fraction C of Ketonic Steroids*

Eluate No	Appearance	Weight mg	M p °C	Compound
1, 2, 3	Oil	16.0		
4	" and crystals	10.0		
5, 6, 7	White needles	14.0	168–170	$\Delta^4$ -Androstenedione-3,17
8, 9, 10	Leaf-like crystals	39.0	143–154	Testosterone
11	Oil	2.0		
12, 13, 14	White needles	12.5	218–221	<i>cis</i> -Testosterone
16	Oil and crystals	30.0		

by mixed melting points. About 4 mg of a substance giving a strong positive test for the 17( $\beta$ )-hydroxyl group was obtained, it formed rosette-shaped crystals which melted at 152°. An additional 4 mg of a compound giving a positive 17( $\beta$ )-hydroxyl test were found which formed cube-shaped crystals melting at 107–110°. The amounts of the latter two substances were too small to permit further purification and characterization. No 17( $\alpha$ )-hydroxyl compounds were detected.

*Other Fractions*—A thorough search of the phospholipide fraction (6.624 gm) gave no evidence for the presence of steroid metabolites and showed no androgenic activity in castrated rats (5).

The non-hydroxy, non-ketonic fraction, 2.680 gm, gave 0.535 gm of unsaponifiable material which was subjected to fractional crystallization and chromatographic analysis. 7 mg of a methanol-insoluble material melting at 60° were isolated which may be identical with the hydrocarbon

frequently isolated from natural sources, particularly urine. Cholesterol, 160 mg and a small amount, 3 mg, of a light red-orange compound, m p 193–193.5°, were isolated. The last compound appeared to be identical with that found in the control experiment.

Negative 17-hydroxyl color tests were obtained in all of the above fractions.

### *Incubation of Testosterone, Experiment 2*

This experiment was conducted under aseptic conditions with sterile buffer, instruments, flask, etc., so as to rule out the possibility of metabolism of the substrate by bacteria. Samples for bacteriological tests were taken from the incubation mixture just before the alcohol was added. Any culture showing a count of over ten colonies<sup>9</sup> per 0.1 ml. on a blood agar plate after 24 hours at 37° was excluded from the series. With the exception just noted the incubation procedure was the same as that previously described. A total of 1000 gm of testosterone was incubated with liver slices from eleven rabbits.

The total fat-soluble residue weighed 9.197 gm, of which 4.781 gm were acetone-soluble. Bioassay of the latter on the castrated rat indicated a 20 per cent loss in activity.

*Recovery of Testosterone*—The ketonic fraction, 0.901 gm, on fractional crystallization from methanol-water mixtures yielded 0.427 gm of testosterone, m p 152.5–154.5°, which was further characterized by carbon and hydrogen analyses, preparation of the benzoate, m p 192°, and mixed melting points.

*Fractionation of Remaining Ketonic Residue*—The pooled residue, 0.391 gm, of liquors from the above crystallizations was subjected to repeated chromatographic separation on a 1:1 magnesium silicate-Celite mixture. The various fractions were pooled in accordance to their reaction to the Zimmermann, Pincus, and the Kagi-Miescher tests. The impure fractions were chromatographed again with aluminum oxide.

*Isolation of  $\Delta^4$ -Androstenedione-3,17*—By these procedures 85 mg of  $\Delta^4$ -androstenedione-3,17, m p 168–170°, were isolated. The substance when mixed with an authentic sample of  $\Delta^4$ -androstenedione-3,17 showed no depression in melting point.

$C_{19}H_{28}O_2$  Calculated, C 79.72, H 9.09, found, C 79.73, H 9.02

The dioxime melted at 141–144°. It did not depress the melting point of an authentic sample of  $\Delta^4$ -androstenedione-3,17 dioxime.

<sup>9</sup> Whenever colonies did appear, they were identified as *Staphylococcus albus* by Dr R. Boak, Department of Public Health.



*Isolation of cis-Testosterone*—A total of 12 mg of *cis*-testosterone, which melted at 213–215°, was isolated. When the substance was mixed with an authentic sample of *cis*-testosterone, m p 218–221°, the melting point was 216–220°. The acetate melted at 111–112° and did not depress the melting point of an authentic sample of *cis*-testosterone acetate.

*Isolation of Other Unidentified Ketones*—A small amount, 0.5 mg, of a strongly adsorbed compound which gave a strong violet color in the Zimmermann reaction was isolated. The needle-shaped crystals were highly colored under crossed polaroids in the microscope, but, in spite of their clean appearance, melted at 250–269° with decomposition.

An additional strongly adsorbed compound was isolated in a small amount, 4 mg. It changed from platelets to needles on the hot stage at 130–140° and melted at 201–203°. A negative 17-hydroxyl test was obtained. The carbon and hydrogen values indicated that an oxygen atom had been introduced into the molecule.

$C_{19}H_{28}O_2$  Calculated, C 75.2, H 9.15, found, C 74.27, H 9.56

$C_{19}H_{26}O_3$  Calculated, C 74.6, H 9.75

*Fractionation of Non-Ketonic Residue*—The non-ketonic residue, 3.191 gm, was saponified with alcoholic potassium hydroxide. The unsaponifiable material, which weighed 1.087 gm, was fractionated by chromatographic analysis on magnesium silicate-Celite mixture. A total of 520 mg of cholesterol was isolated, m p 145.5–147.5°.

Positive color reactions for a 17-hydroxyl compound were obtained in the later fractions, but attempts at isolation of crystalline material were unsuccessful.

#### SUMMARY

Testosterone is metabolized by rabbit liver slices to  $\Delta^4$ -androstenedione-3,17, *cis*-testosterone, and to small amounts of other partially identified steroids including a  $C_{19}H_{28-30}O_3$  compound. None of the known urinary androgens or estrogens could be found. Approximately 20 per cent of the original material could not be accounted for.

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# THE METABOLISM OF L-HISTIDINE

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(Received for publication, May 5, 1947)

Shortly after the discovery of histidine, Abderhalden *et al.* (1) attempted to ascertain the fate of this amino acid when administered to dogs but could demonstrate only an increased excretion of urea and ammonia. However, since these initial experiments were carried out, many investigators have presented evidence in support or refutation of the idea that histidine may be converted into various other metabolites. An extensive review of the subject is not attempted here, but some of the evidence for the possible conversion products will be mentioned briefly. The possibility that histidine and arginine were metabolically interconvertible and played a special rôle in purine synthesis was suggested by Ackroyd and Hopkins (2), based on the observation that when either of these amino acids was restored to a deficient diet of young rats growth was resumed and allantoin excretion returned to normal levels. The indispensability of histidine as a dietary constituent was confirmed by Rose and Cox (3), but arginine, however, was found to be ineffective for growth when substituted for histidine in a deficient diet. The conversion of histidine into creatine was suggested by Abderhalden and Buadze (4). The degradation of histidine to glutamic acid was proposed by Edlbacher and Kraus (5), who isolated glutamic acid after the action of liver histidase upon histidine. The biological decarboxylation of histidine to form histamine was indicated by the increase in the histamine content of guinea pig lungs following the injection of histidine (6). Though ergothioneine, carnosine, and anserine are structurally related to histidine, their metabolic derivation from histidine has not yet been demonstrated.

With the exception of a single isotope experiment which indicated that the imidazole ring of histidine is not synthesized in rats (7), our present knowledge of the metabolism of histidine is based largely on the results of balance experiments on animals or isolated tissues. To obtain further evidence as to the metabolic fate of this amino acid, synthetic L-histidine, containing a

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high concentration of  $N^{15}$  in the ring nitrogen attached to the  $\gamma$ -carbon atom, was fed to rats. A number of amino acids and other nitrogenous substances were isolated and the distribution of the isotope in the organs and some of their components determined.

#### EXPERIMENTAL

Isotopic histidine was synthesized by the procedure of Ashley and Harrington (8, 9).  $N^{15}$  was introduced into the imidazole ring by the condensation of isotopic thiocyanate with  $\gamma$ -ketornithine to form 2-thiol-histidine, which was subsequently oxidized to L-histidine. Since the  $\gamma$ -ketornithine was prepared from naturally occurring histidine, without racemization, the resynthesized histidine had the same steric configuration.

*Isotopic Sodium Thiocyanate*—The procedure of Schulze (10) for the synthesis of thiocyanate was modified to conserve the isotopic ammonia. The modified reaction is  $3N^*H_4NO_3 + 3CS_2 + 2Fe(OH)_3 + 6NaOH = 3NaCN^*S + 2FeS + S + 3NaNO_3 + 12H_2O$ , where the symbol  $N^*$  represents nitrogen with a high concentration of  $N^{15}$ . To a mixture of 25 cc of carbon disulfide and 17 gm of ferric hydroxide in 40 cc of absolute methanol, 10.8 gm of ammonium nitrate, containing 68.4 atom per cent excess  $N^{15}$  in the ammonium ion, were added and the resultant mixture shaken mechanically in a tightly closed bottle. Sodium hydroxide pellets, divided roughly in nine portions totaling 10.8 gm, were added at 2 hour intervals. After being shaken for an additional 24 hours, the mixture was diluted with water and centrifuged. The deposit was washed several times by centrifugation, the combined supernatants were saturated with hydrogen sulfide, filtered, acidified to litmus (not to Congo red), and heated to boiling. On cooling, the solution was neutralized by the addition of dilute sodium hydroxide in an amount equivalent to the hydrochloric acid added, and evaporated to dryness *in vacuo*. The salts were extracted with several portions of absolute ethanol and the extract, containing the sodium thiocyanate, evaporated on a steam bath. The residue was extracted with 100 cc of absolute ethanol and the extract evaporated to dryness. 9.80 gm (90 per cent of theory) of isotopic sodium thiocyanate were obtained. The product was 99 per cent pure as assayed colorimetrically against standardized normal thiocyanate.

*$\gamma$ -Ketornithine*—With minor modifications of the procedures of Ashley and Harrington (9), 200 gm of L-histidine monohydrochloride monohydrate were degraded, in several runs, to 22 gm of  $\gamma$ -ketornithine dihydrochloride. The best over-all yield was about 25 per cent. In this procedure the imidazole ring of the methyl ester of histidine was cleaved by exhaustive benzoylation to form methyl- $\alpha, \gamma, \delta$ -tribenzamido- $\Delta^7$ -pentenoate. Benzoylation by the procedure recommended by Ashley and Harrington gave sticky

products with variable yields of 12 to 25 per cent. Better results were obtained by a two-phase benzoylation with yields of over 60 per cent. To an aqueous solution of the methyl ester hydrochloride of histidine and excess sodium carbonate, a 3-fold excess of benzoyl chloride dissolved in benzene was added and the mixture stirred vigorously for 9 hours at 0°. After filtration, the benzene phase was worked up for the desired product. Hydrolysis of the methyl- $\alpha, \gamma, \delta$ -tribenzamido- $\Delta^7$ -pentenoate in two steps gave  $\gamma$ -ketoornithine dihydrochloride. This was obtained largely as a resin which was not purified further, for owing to its unstable nature such procedures invariably were accompanied by considerable decomposition. Approximately 3 gm. of crystalline product were obtained.

$C_6H_{10}N_2O_2 \cdot 2HCl$  Calculated, N 12.8, Cl 32.4, found, N 13.2, Cl 32.7

*Isotopic Histidine*—By the condensation (9) of 21.0 gm. of  $\gamma$ -ketoornithine with 8.0 gm. of isotopic sodium thiocyanate, 5.94 gm. (32 per cent of theory) of 2-thiohistidine were obtained. This product was oxidized with 80 gm. of ferric sulfate to yield 2.64 gm. (52 per cent of theory) of histidine, which was purified as the monohydrochloride.

$C_6H_7N_3O_2 \cdot HCl \cdot H_2O$

Calculated, N (corrected for isotope content) 20.3, found, N 20.2

"  $N^{15}$  22.8 atom %, found,  $N^{15}$  22.9 atom %

$[\alpha]_D^{25} = +9.69^\circ$  (2.2% in normal HCl)

The  $\alpha$ -amino nitrogen, liberated as ammonia by ninhydrin (11), contained no significant amount of excess  $N^{15}$ .

*Feeding Experiment*—Three adult male rats, having a total weight of 890 gm., were fed an individual daily ration of 15 gm. of a stock diet consisting of 68 per cent corn-starch, 15 per cent casein, 5 per cent yeast, 4 per cent salt mixture (12), 6 per cent Wesson oil, and 2 per cent cod liver oil. After a preliminary feeding period of 2 weeks, a supplement of 1 mM of the isotopic histidine was added to the daily ration of each rat for the experimental period of 3 days. During this period the rats consumed all of the diet and gained less than 2 per cent in weight. The urine and feces were collected daily. At the end of the 3rd day, the rats were killed by exsanguination and the blood collected in oxalate. The bodies were segregated in four groups, namely skin, liver, other internal organs, and carcass. These groups were processed separately and the isotope concentrations of the various components determined (13).

*Excreta*—The daily collection of urine and feces was analyzed for total nitrogen and  $N^{15}$ . Urinary ammonia was adsorbed on permutit, recovered by addition of alkali, and distilled into acid. The urea of an ammonia-free

sample was decomposed by incubation with urease and the ammonia liberated analyzed for N<sup>15</sup>. The results appear in Table I.

The presence of a urinary component having a relatively high N<sup>15</sup> concentration was indicated by the observation that the total nitrogen had a

TABLE I  
*Distribution of Isotope in Excreta*

Day of experiment	Source of nitrogen	N <sup>15</sup> concentration <i>atom per cent excess</i>	Total N <i>mg</i>	Total N <sup>15</sup> <i>mg</i>
1st	Feces	0.298	6.31	0.0188
2nd	"	0.971	8.78	0.0852
3rd	"	1.095	8.35	0.0915
Total		(0.834)*	23.44	0.1955
1st	Whole urine	1.23	59.6	0.734
2nd	" "	1.44	62.2	0.895
3rd	" "	1.62	59.6	0.965
Total		(1.43)*	181.4	2.594
1st	Urea	1.236	46.0	0.569
2nd	"	1.39	46.4	0.645
3rd	"	1.578	46.4	0.731
Total		(1.40)*	138.8	1.945
1st	Ammonia	0.466	3.90	0.0182
2nd	"	0.663	5.02	0.0333
3rd	"	0.752	5.01	0.0376
Total		(0.640)*	13.93	0.0891
Combined urine	Allantoin	0.130		
" "	$\alpha$ -Amino N	1.91-3.43		

\* The figures in parentheses represent the average N<sup>15</sup> concentration of the total excretory partitions, computed as follows

$$\text{Average N}^{15} \text{ atom per cent} = \frac{\text{miliequivalents of total N}^{15}}{\text{miliequivalents total N}} \times 100$$

slightly higher isotope concentration than that of the urea. To obtain some information as to the nature of the high isotopic component, the imidazole content of the urine and the N<sup>15</sup> concentration of the  $\alpha$ -amino nitrogen of the urinary amino acids were determined. Assayed colorimetrically, the imidazole content of the combined 3 day urines, expressed

as histidine, was 22 mg, and was not greater than that observed with rats on a normal stock diet. After removal of urea by incubation with urease, the  $\alpha$ -amino nitrogen was liberated as ammonia by the action of ninhydrin by an adaptation of the procedures of Van Slyke *et al.* (14) and MacFadyen (11). The analyses of several determinations varied from 1.91 to 3.43 atom per cent excess  $N^{15}$ , the variations being apparently due to differences in the alkali concentration employed and to prolongation of the aspiration period for the removal of ammonia. This effect was not investigated further because of the desire to isolate the purine end-product, allantoin, from the limited volume of urine remaining. Allantoin was isolated by the procedure of Wiechowski (15), m.p. 235°.

$C_4H_6N_4O_2$  Calculated, N 35.4, found, N 35.0

*Blood*—The oxalated blood was centrifuged and the proteins of the plasma were precipitated with 6 per cent trichloroacetic acid and hydrolyzed in hydrochloric acid. Samples of the plasma non-protein fraction and protein hydrolysate were digested in concentrated sulfuric acid for  $N^{15}$  analysis. The cells were washed with isotonic saline, hemolyzed by the addition of water, and the cell residues deposited by centrifugation. Hemun was obtained as an amorphous precipitate by the addition of the hemolysate to a solution of acetic acid and sodium chloride (16). After two reprecipitations, the material was analyzed for  $N^{15}$ . The residual cell hemolysate was hydrolyzed in hydrochloric acid, an aliquot removed for total nitrogen analysis, and the balance processed for the isolation of histidine as the hydrochloride (17).

$C_6H_9N_3O_2 \cdot HCl \cdot H_2O$  Calculated, N 20.1, found, N 19.6,  $N^{15}$  0.504 atom %

The  $\alpha$ -amino nitrogen, liberated by ninhydrin (11) had an  $N^{15}$  concentration of 0.031 atom per cent excess. Since the  $\delta$ -nitrogen of the imidazole ring should contain no excess isotope, the concentration of the  $\gamma$ -nitrogen was computed as  $(3 \times 0.504) - 0.031 = 1.481$  atom per cent excess  $N^{15}$ . The results are given in Table II.

*Creatinine*—Muscle creatine was isolated as creatinine picrate from an alcoholic extract of half of the minced carcass (18) and purified as creatinine zinc chloride.

$(C_4H_7N_3O)_2 \cdot ZnCl_2$  Calculated, N 23.2, found, N 22.6

A sample of the creatinine zinc chloride was refluxed in a 10 per cent solution of barium hydroxide for 24 hours and the amidine nitrogen, liberated as ammonia, swept over into dilute acid by a stream of nitrogen gas. It amounted to 96 per cent of the calculated quantity. The isotope content of the sarcosine moiety was determined by removal of the barium with



TABLE II  
*Distribution of Isotopic Nitrogen in Blood*

Source of nitrogen		N <sup>15</sup> concentration	Total N	Total N <sup>15</sup>
		atom per cent excess	m eq	m eq
Plasma	Non-protein N	0 780	0 49	0 0039
	Protein N	0 612	10 8	0 0662
Erythrocytes	" "	0 096	19 6	0 0183
	Cell residues	0 051	21 3	0 0109
	Hemin	0 042		
	Histidine	0 504		
	" α-N	0 031		
	" γ-N	1 48*		

\* Calculated as atom per cent N<sup>15</sup> of γ-N = (atom per cent N<sup>15</sup> of histidine × 3) - atom per cent N<sup>15</sup> of α-N

TABLE III  
*Distribution of Isotopic Nitrogen in Organ Components*

Source of nitrogen	Carcass	Liver	Internal organs
	atom per cent excess	atom per cent excess	atom per cent excess
Histidine	1 264	4 50	
" α-N	0 033	0 058	
" γ-N	3 759*	13 44*	
Glutamic acid	0 078	0 697	
Aspartic "	0 060	0 574	
Arginine	0 023	0 334	
" amidine N	0 043	0 651	
" ornithine N	0 012	0 064	
Tyrosine	0 021	0 103	
Proline	0 017		
Creatinine	0 027		
" amidine N	0 033		
" sarcosine N	0 017		
Adenine		0 217	0 109†
Guanine		0 194	0 096
Amide N	0 060	0 316	
Total protein N	0 090	0 479	0 110
Non-protein N			
Trichloroacetic acid extract	0 220	0 668	0 269
Alcohol extract		0 296	0 243

\* See foot-note to Table II

† Calculated from the atom per cent N<sup>15</sup> of adenine picrate (see the text)

excess sulfuric acid and digesting the concentrated filtrate The results appear in Table III

*Carcass Non-Protein and Total Protein Nitrogen*—The untreated half of the minced carcass was extracted with 6 per cent trichloroacetic acid to remove the non-protein nitrogen fraction and the residual tissue hydrolyzed in hydrochloric acid. Aliquots of the trichloroacetic acid extract and the hydrolysate were analyzed for nitrogen content and  $N^{15}$ . Tyrosine, histidine, arginine, proline, glutamic acid, aspartic acid, and amide nitrogen were isolated by the usual methods. The analytical values appear in Table IV and their  $N^{15}$  concentrations in Table III.

*Purines of Liver and Internal Organs*<sup>1</sup>—The liver and internal organ fractions were processed separately for the isolation of adenine and guanine. The minced tissue was extracted with 6 per cent trichloroacetic acid, washed with ethanol, and dried. Aliquots of the trichloroacetic acid and ethanol extracts were analyzed for  $N^{15}$ . The dried tissue was refluxed for 1 hour in normal hydrochloric acid in 50 per cent formic acid, liberating the readily hydrolyzed purines. After removal of the bulk of the acids by

TABLE IV  
*Nitrogen Analyses of Isolated Carcass Components*

Components analyzed	N calculated	N found
	<i>per cent</i>	<i>per cent</i>
Tyrosine	7.74	7.71
Histidine 3,4-dichlorobenzenesulfonate	6.91	6.94
Proline	12.2	12.0
Glutamic acid hydrochloride	7.63	7.41
Aspartic “	10.5	10.5
Arginine monohydrochloride	26.5	26.4

vacuum distillation, the purines were precipitated from a hot solution of the syrup by the addition of a suspension of cuprous oxide. The precipitate was dissolved in hot 40 per cent trichloroacetic acid, boiled for 45 minutes, the pH adjusted to 5.0 with normal sodium citrate, and the purines reprecipitated by adding cuprous oxide. The precipitate was suspended in normal hydrochloric acid, copper was removed with hydrogen sulfide, and the filtrate concentrated by vacuum distillation. Guanine was precipitated from the concentrated aqueous solution by adjusting the pH to 5.0 and was recrystallized as the sulfate.

$C_5H_5N_5O \cdot H_2SO_4 \cdot 2H_2O$

Calculated N 32.1,

Found Liver guanine N 32.2, internal organ guanine N 31.0

<sup>1</sup> The procedure for the isolation of purines was kindly suggested by Dr. Samuel Graff, College of Physicians and Surgeons, Columbia University, New York.

Adenine picrate was precipitated from the mother liquor by the addition of saturated picric acid. Adenine picrate from the liver fraction was dissolved in normal hydrochloric acid, picric acid extracted with ether, and the adenine hydrochloride obtained from the concentrated aqueous solution recrystallized.

$C_8H_8N_6 \cdot HCl \cdot \frac{1}{2}H_2O$  Calculated, N 38.8, found, N 36.8

Because very little adenine picrate was obtained from the internal organ fraction, it was analyzed directly after reduction with phosphorus and hydriodic acid.

$C_8H_8N_6 \cdot C_6H_3N_3O_7 \cdot H_2O$  Calculated, N 30.8, found, N 31.2,  $N^{15}$  0.068 atom %

The  $N^{15}$  concentration of the adenine was computed as  $0.068 \times 8/5 = 0.109$  atom per cent excess  $N^{15}$ .

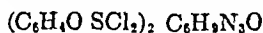
Soluble copper was precipitated from the hydrolysates of the liver and internal organ fractions with hydrogen sulfide, and the filtrates hydrolyzed in 20 per cent hydrochloric acid. Aliquots were taken for the determination of total nitrogen and  $N^{15}$ . The results appear in Table III.

*Liver Amino Acids*—From the liver hydrolysate, amide nitrogen and several amino acids were isolated by similar procedures used for the carcass constituents. The analytical values appear in Table V and the isotope concentrations are given in Table III. The low value of the nitrogen analysis of glutamic acid was attributed to the presence of sodium chloride introduced during a preliminary isoelectric precipitation from hydrochloric acid solution by the addition of sodium hydroxide. The amount of glutamic acid remaining was too small for further purification. If, as we believe, the low nitrogen value results from contamination by sodium chloride, the  $N^{15}$  values will not be in error, for no nitrogen-containing impurity is involved.

*Skin*—The rat skins were hydrolyzed in hydrochloric acid and a sample taken for total nitrogen and  $N^{15}$  analysis (Table VII).

*Histidine Content of Liver and Carcass*—In a separate experiment, the histidine content of liver and carcass of rats on a stock diet was determined by the isotope dilution method (19). The combined livers of three adult rats and a single carcass were minced separately, extracted with 6 per cent trichloroacetic acid, and hydrolyzed for 24 hours in 20 per cent hydrochloric acid. Isotopic histidine, containing 22.9 atom per cent excess  $N^{15}$  (for all 3 N atoms), was added to each solution before hydrolysis, 0.0374 mM to the liver fraction and 0.163 mM to the carcass fraction. Aliquots of each hydrolysate were taken for total protein nitrogen, yielding 60.7 milliequivalents for liver and 28.4 milliequivalents for carcass hydrolysate. Excess hydrochloric acid was removed by vacuum distillation, and a solution of the syrup made alkaline with barium hydroxide. Insoluble

humins was filtered off and aliquots analyzed for nitrogen content and  $N^{15}$ . Amide nitrogen of the hydrolysate, evolved as ammonia, was aspirated into dilute acid by a stream of nitrogen gas for 12 hours and was analyzed for nitrogen content and  $N^{15}$ . Barium was removed from the hydrolysate with sulfonic acid, and histidine was precipitated with alcoholic mercuric chloride (17) and purified as the dichlorobenzenesulfonate



Calculated N 6.91

Found Liver N 6.79,  $N^{15}$  0.788 atom %, carcass N 6.82,  $N^{15}$  0.940 atom %

The amount of histidine in each hydrolysate was computed from the  $N^{15}$  concentration and amount of the isotopic histidine added, and the  $N^{15}$  concentration of the isolated histidine in liver  $((22.9/0.788) - 1) \times 0.0374 = 1.05$  mM, and in carcass  $((22.9/0.940) - 1) \times 0.163 = 3.80$  mM. The percentage of histidine nitrogen in the total protein nitrogen was  $(1.05 \times$

TABLE V  
*Nitrogen Analyses of Isolated Liver Components*

Components analyzed	N calculated	N found
	per cent	per cent
Tyrosine	7.74	7.71
Histidine 3,4-dichlorobenzenesulfonate	6.91	6.97
Glutamic acid hydrochloride	7.63	6.49
Aspartic "	10.5	10.3
Arginine monohydrochloride	26.5	26.0

$3 \times 100)/60.7 = 5.2$  per cent for liver and  $(3.80 \times 3 \times 100)/284 = 4.0$  per cent for carcass

Appreciable amounts of  $N^{15}$  were found in the humins and amide nitrogen fractions of each hydrolysate

Liver humin	3.67 m eq	$N \times 0.020$	atom % $N^{15} = 7.34 \times 10^{-4}$	m eq $N^{15}$
" amide N	2.87	" " $\times 0.014$	" % " $= 4.02 \times 10^{-4}$	" "
Carcass humin	5.07	" " $\times 0.011$	" % " $= 5.58 \times 10^{-4}$	" "
" amide N	6.11	" " $\times 0.006$	" % " $= 3.67 \times 10^{-4}$	" "

The  $N^{15}$  in these fractions was derived from the added isotopic histidine and indicates a degradation or adsorption of histidine. The quantity and percentage of the isotopic histidine supplement, corresponding to the  $N^{15}$  found in each fraction, was computed from the data above

Liver humin	$(7.34 \times 10^{-4}) / (0.229 \times 3) = 10.7 \times 10^{-4}$ mM histidine	(2.9%)
" amide N	$(4.02 \times 10^{-4}) / (0.229 \times 3) = 5.85 \times 10^{-4}$ "	(1.6%)
Carcass humin	$(5.58 \times 10^{-4}) / (0.229 \times 3) = 8.12 \times 10^{-4}$ "	(0.5%)
" amide N	$(3.67 \times 10^{-4}) / (0.229 \times 3) = 5.34 \times 10^{-4}$ "	(0.4%)

The total quantity of isotopic histidine lost in these fractions was  $16.6 \times 10^{-4}$  mm (4.4 per cent) in liver hydrolysate and  $13.5 \times 10^{-4}$  mm (0.8 per cent) in carcass hydrolysate

### DISCUSSION

*Recovery of Isotope*—During the feeding period the rats consumed 9 mm of isotopic histidine containing 68.4 atom per cent excess  $N^{15}$  in the  $\gamma$ -nitrogen, or a total of 6.15 miliequivalents of  $N^{15}$ . Only 82 per cent of the isotope was accounted for in the fractions analyzed (Table VI). In the preparation of the tissue fractions, the gastrointestinal contents were washed out and discarded without determining the  $N^{15}$  content. Since the diet of the preceding day contained 33 per cent of the administered isotope, this fraction may account for part or all of the missing 18 per cent. The feces gave a negative diazo test for histidine and contained only 3 per cent

TABLE VI

*Distribution of Isotopic Nitrogen in Excreta and Animal Body*

The diet contained a total of 6.15 miliequivalents of  $N^{15}$

Source of nitrogen	$N^{15}$ in fraction	Amount recovered
	mg	per cent
Feces	0.20	3
Urine	2.59	42
Non-protein N	0.32	5
Total protein N	1.98	32
Total $N^{15}$ recovered	5.09	82

of the total  $N^{15}$ , indicating that the administered histidine was well absorbed

*Urinary Ammonia and Urea*—In all cases in which amino acids containing excess  $N^{15}$  in the  $\alpha$ -amino group have been fed to animals in this laboratory (20-23), the isotope concentration of urinary ammonia was found to be consistently higher than that of urea. On the other hand, in this experiment the  $N^{15}$  concentration of the urinary ammonia was less than half of that of the urea (Table I). This difference in the relative isotope concentrations of urinary components may be attributed to either the formation of urinary ammonia from  $\alpha$ -amino acids or a specific utilization of degraded imidazole nitrogen for the synthesis of urea. The formation of urinary ammonia from the deamination of  $\alpha$ -amino acids by kidney deaminases offers a ready explanation for the higher isotope concentration found in ammonia in those experiments in which the excess  $N^{15}$  was located in the  $\alpha$ -amino group of the amino acid fed. Although the nitrogen from moder-

ately large amounts of histidine administered in balance experiments is excreted largely as urea (1), the existing theories for the synthesis of urea do not lend support to a hypothesis for a specific conversion of imidazole nitrogen to urea. Urea might conceivably be produced by a direct cleavage of the imidazole ring or through the intermediate formation of citrulline, by a splitting of the ring between the  $\gamma$ -carbon and the adjacent nitrogen. Citrulline may form urea either by way of the arginine cycle of Krebs and Henseleit (24) or by degradation to glutamic acid as proposed by Bach (25). If the conversion of isotopic imidazole nitrogen to urea proceeded directly through citrulline and arginine, a relatively high concentration of  $N^{15}$  would be expected in liver arginine. However, this was not observed. Glutamine formed from the degradation of histidine by the action of liver histidase (26) may play a special rôle in the production of urea (27) and urinary ammonia (28). However, glutamine formed by this mechanism from isotopic histidine would contain no excess  $N^{15}$ , for only the normal  $\alpha$ - and  $\delta$ -nitrogens of histidine are retained in the conversion, and consequently the urea or ammonia subsequently produced would also lack excess  $N^{15}$ .

*Urinary Degradation Product of Histidine*—The presence of a component in the urine of relatively high isotope concentration was indicated by the observation that the  $N^{15}$  concentration of the average total nitrogen (1.43 atom per cent) was significantly higher than that of urea (1.40) and ammonia (0.64) (Table I), which together made up 84 per cent of the urinary nitrogen. This component was not due to contamination of the urine with the dietary isotopic histidine supplement, for the urine contained only traces of spilled food. The imidazole content, assayed as 22 mg of histidine, even if it contained the high  $N^{15}$  concentration of administered histidine, would influence only slightly the  $N^{15}$  concentration of the total nitrogen.

The  $N^{15}$  concentration of the  $\alpha$ -amino nitrogen liberated from the urine by the action of ninhydrin ranged from 1.91 to 3.43 atom per cent excess  $N^{15}$ . The most probable source of the urinary amino acids is the plasma, and the  $N^{15}$  concentration of the amino acids by the plasma may be expected to be higher than that found in the tissue proteins. However, it would be striking indeed if the average  $N^{15}$  value, as represented by the results obtained for the urinary amino acids, were almost 5 times that determined for the most metabolically active amino acid of the liver, namely glutamic acid with an  $N^{15}$  concentration of 0.697 atom per cent excess. It is not improbable that a degradation product of histidine, in which the imidazole ring had been ruptured and the  $\gamma$ -nitrogen subject to cleavage by ninhydrin or alkali, was excreted in the urine and thereby contributed its high  $N^{15}$  content to the  $\alpha$ -amino nitrogen determination. A urinary degradation product of histidine is suggested by the observation of Eaton and Doty (29),

that following the administration of moderately large amounts of histidine to a dog a considerable amount of an unidentified non-imidazole substance was found in the urine. In rats after the injection of histidine there was an increase in the amino nitrogen of the urine which exceeded the increase of imidazole nitrogen (30). In the action of liver histidase on histidine, an intermediate product was obtained which was unstable to alkali, the amount of ammonia liberated being dependent upon the alkali concentration (5). Such degradation products in the urine may possibly account for the high  $N^{15}$  values obtained.

*Isotope Distribution of Organ Proteins*—The concentration of  $N^{15}$  in the various organ proteins (Table VII) was not uniform and may depend on (a) the concentration of  $N^{15}$  in the reactive substances in the environmental

TABLE VII  
*Distribution of Isotope in Tissue Proteins*

Tissue proteins	$N^{15}$ concentration	Total N	Total $N^{15}$	Fraction of total $N^{15}$ in proteins
	atom per cent excess	mg	mg	per cent
Erythrocytes				
Cell residues	0.051	21.3	0.0109	1
Hemoglobin	0.096	19.6	0.0188	1
Plasma	0.612	10.8	0.0662	3
Liver	0.479	50.9	0.244	12
Internal organs	0.110	72.3	0.0800	4
Carcass	0.090	1057	0.95	48
Skin	0.089	689	0.61	31
Total $N^{15}$ in tissue proteins			1.979	100

plasma, (b) the concentration of exchangeable components in the organ proteins and environmental plasma, and (c) the relative chemical activity or rate of protein turnover. In contrast with the plasma proteins, which had the highest concentration of  $N^{15}$  of all the tissues investigated, the proteins of the erythrocytes displayed a relatively low value, which reflects the slow rate of synthesis of the red blood cell. The  $N^{15}$  concentration of the liver proteins was over 5 times that found in carcass and skin and may be attributed both to its great metabolic activity and to its favorable location for reaction with dietary histidine absorbed from the intestine by way of the portal vein. Although the proteins of the carcass and skin had the lowest  $N^{15}$  concentrations, these organs, because they contain the bulk of the body proteins, contained 80 per cent of the total  $N^{15}$  in the animal. The pooled internal organs were a mixture of several kinds of tissues, which

differ in function and composition, and the  $N^{15}$  concentration obtained was an average figure and does not indicate the relative activity of the separate organs. When other isotopic amino acids, as L-leucine (20) or L-proline (23), were fed to rats, the  $N^{15}$  concentration of several of the internal organs, notably the intestinal wall and kidney, differed little from that obtained for liver. The low value obtained here for the mixture of internal organs may indicate a very slow rate of exchange in some of the other internal organs.

*Replacement of Organ Histidine*—The high  $N^{15}$  concentrations of isolated histidine (Table III) are referable to the imidazole nitrogen, for the  $N^{15}$  concentration of the  $\alpha$ -amino nitrogen was very low. Since the imidazole group is not synthesized in the animal (7), the isotope of the imidazole group of tissue histidine was derived directly from the dietary supplement and the  $N^{15}$  must be, therefore, solely in the  $\gamma$ -nitrogen. In addition to the 1 mM of isotopic histidine supplement, the daily ration of each rat contained 2.250 gm of casein with a histidine content of 3.0 per cent (31), or 0.435 mM of histidine, and 760 mg of yeast with a histidine content of 1.03 per cent (32), or 0.050 mM of histidine, making a total of 1.48 mM. Since the  $\gamma$ -nitrogen of the histidine supplement had a  $N^{15}$  concentration of 68.4 atom per cent excess, the  $N^{15}$  concentration of the  $\gamma$ -nitrogen in the total dietary histidine was  $68.4 \times 1.00/1.48 = 46.2$  atom per cent excess  $N^{15}$ . The percentage of histidine replaced in the tissues can be computed from this figure and the  $N^{15}$  concentration of the  $\gamma$ -nitrogen of the isolated histidine. In liver,  $13.4/46.2 \times 100 = 29$  per cent of the total histidine was replaced by dietary histidine in 3 days. In a similar manner, it was computed that at least 8 per cent of carcass and 3 per cent of erythrocyte histidine had been replaced. When dietary L-leucine was fed, 24 per cent of liver leucine and 7 per cent of carcass leucine were replaced within the same experimental period (20). The close agreement of the turnover rate of histidine and leucine in the proteins of the liver and carcass may indicate that the regeneration of these proteins occurs by a process in which the entire molecule is synthesized from amino acids rather than by replacement of amino acid residues one at a time. The replacement rate discussed above is in accord with the finding that half of the total nitrogen of liver proteins is replaced by the nitrogen of the diet and other proteins in 7 days (22). Shemin and Rittenberg have recently measured the life span of the red blood cell in the human and found it to be about 127 days (33). The replacement rate found here for hemoglobin histidine indicates that about 1 per cent of the hemoglobin is synthesized per day in the red blood cell of the rat and is in agreement with their value.

The absolute amount of histidine replaced in the liver can be computed from the results of the determination for the percentage of histidine nitrogen in total liver protein nitrogen obtained by the isotope dilution method (see



"Experimental") It is the product of one-third of the concentration of histidine nitrogen, the total liver protein nitrogen (Table VII), and the concentration of replaced histidine  $\frac{1}{3} \times 0.052 \times 50.9 \times 0.29 = 0.256$  mM. The amount of  $N^{15}$  in the replaced histidine is computed as  $0.256 \times 0.464 = 0.119$  milliequivalent, and its percentage of the total  $N^{15}$  of the liver (Table VII) is  $0.119 \times 100/0.244 = 49$  per cent. By a similar computation, it can be shown that 1.17 milliequivalents of histidine had been replaced in the carcass tissue and contained 57 per cent of total  $N^{15}$  (Table VII). The balance of the isotope was distributed among all the other constituents of the respective organs. The replaced histidine of the liver and carcass was equivalent to 2 and 9 per cent, respectively, of the total histidine of the diet.

*A Criterion for Possible Conversion Products*—Measurable amounts of  $N^{15}$  were found in every component isolated from the tissues (Tables II and III), indicating a non-specific utilization of the  $\gamma$ -nitrogen of histidine following its metabolic degradation. The relative  $N^{15}$  concentrations of the amino acids (Table III) other than histidine are not unlike those observed after feeding isotopic ammonium citrate (16, 18, 34) or other amino acids (20-23). Since the imidazole nitrogen of histidine is not subject to nitrogen exchanges, the occurrence of  $N^{15}$  in all the other components must be due either to the direct conversion of histidine or to synthetic procedures and nitrogen exchanges subsequent to the liberation of the  $N^{15}$  from the imidazole ring. Such nitrogen exchanges are supported by the mechanisms of transamination of Braunstein and Kritzman (35) and the deamination and reamination systems of Knoop and Oesterlin (36) and von Euler *et al* (37). Metabolites of which histidine is an immediate precursor may be expected to contain a high  $N^{15}$  concentration provided that (a) the  $N^{15}$  remains attached to the carbon chain in the conversion, (b) the metabolite is not subject to rapid nitrogen exchanges, producing a marked dilution of the isotope, and (c) the conversion is sufficiently rapid for the  $N^{15}$  concentration to reach levels higher than that observed following the administration of isotopic substances which are not specific precursors.

In the utilization of  $N^{15}$  in tracer experiments there is no basic nitrogen fraction representative of a common "nitrogen pool," having a uniform isotope concentration similar to that of "body water" in experiments in which deuterium is employed as a marker. In each organ, however, certain fractions, such as total protein nitrogen and non-protein nitrogen, whose  $N^{15}$  concentrations are an average of all the components they contain, may be selected as a basis for comparison of the relative  $N^{15}$  concentrations of possible conversion products. Thus, it may be possible to indicate the direct conversion of histidine into other compounds, if it can be demonstrated that the relative  $N^{15}$  concentration of the component under con-

sideration, when compared to such average nitrogen pools, is consistently greater than that obtained by feeding isotopic substances which are not immediate precursors. In Table VIII, in addition to the  $N^{15}$  concentrations of the total protein nitrogen and non-protein nitrogen, the values for amide nitrogen and urea are also employed as comparative bases. Amide nitrogen is an ammonia type of moiety with an active turnover rate, and urea, since it is produced in relatively large amounts in the liver from ammonia, can be taken as a favorable basis for liver components with the exception of arginine. In Table VIII a series of ratios of the  $N^{15}$  concentrations of glutamic acid of the liver is compared with similar ratios obtained from other experiments in which the isotopic dietary supplement was not considered an immediate precursor of the glutamic acid. In each column, the ratios obtained in the comparable experiments are similar to that in the histidine experiment. From this evidence histidine cannot be considered a specific precursor for glutamic acid, for the  $N^{15}$  contributed to this com-

TABLE VIII

*Ratio of Isotope Concentration in Glutamic Acid to Selected Nitrogen Fractions*

Labeled compound fed	Glutamic acid	Glutamic acid	Glutamic acid	Glutamic acid
	Total protein N	Non protein N	Urea	Amide N
L-Histidine	1.45	1.04	0.50	2.20
L-Leucine (20)	1.99	1.49	0.76	2.38
Ammonium citrate (16)	2.09	1.39		2.09

pound by histidine is certainly not greater than that observed when other isotopic amino acids are administered.

The degradation of histidine to glutamic acid by the action of liver enzymes, according to Edlbacher and Neber (26), may occur by two hypothetical mechanisms. In the main route of the hypothesis, the imidazole ring is ruptured by liver histidase, followed by the loss of the  $\gamma$ -nitrogen. In such a mechanism the glutamic acid formed would contain no  $N^{15}$ . In the alternative route, a smaller proportion of the histidine is first deaminated to urocanic acid in which the imidazole ring is subsequently cleaved by urocanase to yield glutamic acid, the  $\gamma$ -nitrogen of histidine being retained as the  $\alpha$ -amino nitrogen of glutamic acid. However, owing to the rapid turnover rate of the  $\alpha$ -amino nitrogen of glutamic acid, it is doubtful whether the glutamic acid formed by such a conversion would retain a sufficiently high  $N^{15}$  concentration to establish its derivation from histidine. This view is confirmed by the biological conversion of proline, synthesized with both deuterium and  $N^{25}$ , into glutamic acid (23). This conversion was demonstrated by the high deuterium content of the isolated

glutamic acid, but the  $N^{15}$  concentration of the glutamic acid with respect to that of aspartic acid was not greater than the ratio obtained after feeding isotopic leucine (20). Apparently, if the  $N^{15}$  were not lost in the initial conversion of proline into glutamic acid, then the concentration was decidedly reduced by the rapid nitrogen exchanges of the  $\alpha$ -amino group of the glutamic acid formed. Glutamic acid, synthesized by transamination mechanisms from  $\alpha$ -ketoglutaric acid available as an intermediate in carbohydrate metabolism, would also contribute to the dilution of  $N^{15}$  of the conversion product. From these considerations, the findings of this experiment do not exclude the mechanism proposed by Edlbacher. To test such a mechanism, histidine synthesized with the carbon isotope would be required. The metabolic conversion of ornithine into glutamic acid has been demonstrated with deuterium as a label (38).

Although histidine might conceivably be degraded to aspartic acid, the same comparative ratios of the  $N^{15}$  concentrations as calculated for glutamic acid do not lend support to such a hypothesis. The same holds for arginine, the relative isotope concentrations are not sufficiently high to indicate any specific contribution of isotope from histidine. The relatively higher  $N^{15}$  value of the amidine nitrogen is attributed to the participation of arginine in the synthesis of urea. In experiments of longer duration, the amidine nitrogen of arginine and urea had approximately equal  $N^{15}$  concentrations (22). The utilization of ornithine (39) and proline (23) for the synthesis of arginine in the rat has been demonstrated by means of deuterium as a tracer.

Both tyrosine and proline undergo comparatively slow nitrogen exchanges or syntheses as indicated by their low isotope concentrations. Histidine does not contribute  $N^{15}$  directly to the formation of these components, for the ratios of their  $N^{15}$  concentrations with respect to basic nitrogen fractions are not greater than that observed when other isotopic amino acids or ammonia was administered.

The low  $N^{15}$  concentration of creatinine, and its ratio with respect to the  $N^{15}$  concentrations of total protein nitrogen and non-protein nitrogen of carcass, compared to similar ratios obtained after feeding isotopic ammonium citrate do not indicate any direct conversion of histidine nitrogen into creatine.

Allantoin (Table I), the metabolic end-product of purines in the rat, has an  $N^{15}$  concentration intermediate between those of the purines of the liver and of the other internal organs respectively (Table III) and may be taken as representative of the isotope values of total body purines. Barnes and Schoenheimer (34) found that following the feeding of isotopic ammonium citrate to rats the  $N^{15}$  concentration of allantoin was approximately equal to that of the tissue purines. The ratio of the  $N^{15}$  concentration of allan-

toin, with respect to that of urea, is not greater than that obtained with isotopic ammonium citrate. It can be concluded that the imidazole ring of histidine is not utilized for the direct synthesis of purines to any extent.

*Hemin*—The low value of  $N^{15}$  obtained for hemin is in agreement with the low values of isotopic nitrogen found in hemin after feeding isotopic ammonium citrate (16), proline (23), or leucine (20) and gives no indication that histidine plays a special rôle in its synthesis.

*Humic and Amide Formation*—In the determination of the histidine content of rat liver and carcass by the isotope dilution method, humic and amide nitrogen, obtained from the hydrolysates to which isotopic histidine had been added before hydrolysis, were found to contain  $N^{15}$  in appreciable amounts. On refluxing histidine in concentrated hydrochloric acid in the presence of carbohydrate about 2 per cent of the histidine nitrogen was found in humic (40) and was attributed to adsorption rather than to modification of the histidine molecule (41). On the other hand, the presence of  $N^{15}$  in the amide nitrogen fraction must be due to decomposition. Under the vigorous conditions of acid hydrolysis employed here, it is evident that the imidazole group of histidine was slowly disrupted.

#### SUMMARY

1 L-Histidine was synthesized with a high concentration of  $N^{15}$  in the  $\gamma$ -nitrogen of the imidazole ring.

2 The isotopic histidine was added to the stock diet of three adult rats for 3 days and was well absorbed. Only 3 per cent of the  $N^{15}$  was excreted in the feces, 42 per cent in the urine, and the major part of the balance incorporated in the tissue proteins. The highest concentrations of  $N^{15}$  were found in the blood plasma and liver proteins, while that in the muscle, skin, internal organ, and erythrocyte proteins was relatively low.

3 The isotope concentration of urinary ammonia, with respect to that of urea, was lower than that observed in experiments in which the  $N^{15}$  was situated in the  $\alpha$ -amino nitrogen of the dietary amino acid. This dissimilarity in the distribution of the  $N^{15}$  is attributed to the rôle of  $\alpha$ -amino nitrogen in the formation of urinary ammonia.

4 A relatively high  $N^{15}$  concentration was obtained in the determination of the  $\alpha$ -amino nitrogen of urinary amino acids and may possibly indicate the presence of a degradation product of histidine.

5 During the 3 day experimental period, the minimal amount of histidine replaced in liver was 29 per cent, in carcass 8 per cent, and in erythrocytes 3 per cent. Of the total  $N^{15}$  present in the respective organs, 49 per cent of the liver  $N^{15}$  and 57 per cent of the carcass  $N^{15}$  were present in the replaced histidine. The replaced histidine of the liver and carcass was 2 and 9 per cent, respectively, of the total histidine consumed in the diet.

6 From the relative concentrations of the isotope in the components isolated, it was apparent that the imidazole nitrogen was not selectively utilized upon rupture of the ring, but was redistributed in a manner similar to that of the nitrogen of ammonium salts or amino acids, which are not specific precursors. There is no evidence that histidine is a precursor of glutamic acid, arginine, creatine, or purines.

7 The concentration of histidine nitrogen in the total protein nitrogen of the whole organ of the rat, as determined by the isotope dilution method, is 5.2 per cent for liver proteins and 4.0 per cent for carcass proteins.

8 Evidence for the partial decomposition of histidine, during acid hydrolysis of normal proteins to which isotopic histidine has been added, is presented by the finding of  $N^{15}$  in the amide nitrogen fraction.

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# THE EFFECT OF INSULIN UPON THE SYNTHESIS OF GLYCOGEN BY RAT DIAPHRAGM IN VITRO

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(Received for publication, May 24, 1947)

Numerous experiments have been reported in an attempt to demonstrate an effect of insulin upon metabolic reactions of isolated tissue *in vitro*. Among these the effect of insulin in increasing the synthesis of glycogen from glucose by rat diaphragm, first reported by Gemmill (1), is in some respects unique, first, because the experimental technique of the demonstration is extremely simple, and second, because of the relative constancy of the results obtained. For these reasons, the studies of Gemmill were extended by a survey of the influence of various factors upon the reaction. These experiments form the substance of this paper.

**Methods**—White rats were used and were fasted 24 hours unless otherwise indicated. Following decapitation the diaphragms were removed with minimum trauma as follows. The abdomen is opened and the diaphragm exposed. A small nick is made in the central fibrous portion, whereupon the diaphragm balloons out. With a small iris scissors each hemidiaphragm is removed, being handled at the edges only with a small forceps. With care, the dissection on the right side around the great vessels is easily made without bleeding. Without preliminary washing, the hemidiaphragm is suspended upon a hook and weighed on the torsion balance and then placed directly into the medium contained in a conical respirator of the Warburg type.

**Buffer**—Unless otherwise stated this was sodium phosphate 0.040 M,  $MgCl_2$  0.005 M, NaCl 0.080 M, glucose 0.2 per cent, pH 6.8 (by glass electrode), final volume 3 ml.

The respirators were attached to Warburg manometers, gassed with 100 per cent of oxygen, and equilibrated usually for 2 hours at 38°. The respiratory period was terminated by the addition of acid, and the hemidiaphragm removed and analyzed for glycogen by a method essentially that of Good, Kramer, and Somogyi (2).

Oxygen and  $CO_2$  exchange were calculated in the usual way. Lactic acid was determined by the Barker-Summerson method (3).

The insulin used was an amorphous zinc-free preparation supplied by Eli Lilly and Company (assay, 22 units per mg).

**Method of Recording Data**—For convenience of comparison with oxygen



done at either of these concentrations. The lower limit at which an insulin effect can be demonstrated is approximately 0.001 to 0.003 unit per ml.

*Effect of Insulin upon Glycogen Synthesis at Varying pH*—This was studied by varying the initial pH of the phosphate-saline medium (Fig. 4)

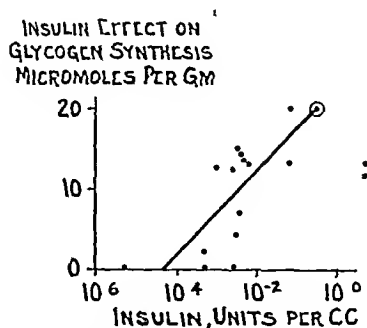


FIG. 3 Insulin effect on glycogen synthesis *in vitro* by rat diaphragms at varying insulin concentrations. The circle indicates the mean value of many single determinations.

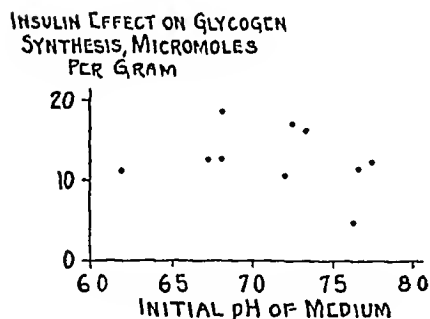


FIG. 4

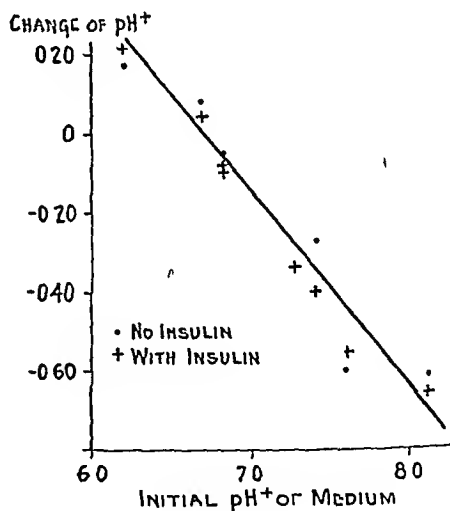


FIG. 5

FIG. 4 Insulin effect on glycogen synthesis *in vitro* by rat diaphragms at varying pH.

FIG. 5 Rat diaphragms in glucose-phosphate-saline medium, the change of pH of the medium in 2 hours as a function of the initial pH.

Between pH 6.3 and 7.6 the insulin effect on glycogen synthesis is essentially the same. However, the pH had considerable effect upon the metabolism in other respects. For example (Fig. 5), when the change of pH was measured at the end of the 2 hour equilibration period, there was found a

linear relation between initial pH and change of pH. The greater the initial pH, the greater was the decrease of pH. At approximately pH 6.8, the pH change was practically zero, at lower initial pH values, there was an increase rather than a decrease of pH. These changes were found to be directly correlated with formation of lactic acid, which accounted for the greater part of the acid changes. Since it was best in balance studies to avoid increases of lactic acid, the pH value of 6.8 was selected for most of the work reported here.

*Effect of Variation of Phosphate Concentration*—The sodium phosphate concentration of the medium was varied from 0 to 0.140 M. The pH (glass electrode) was maintained constant at 6.8 and the osmolar concentration

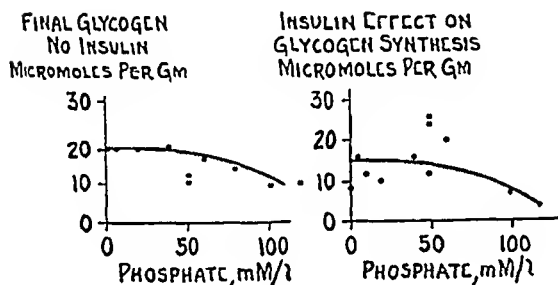


FIG 6

FIG 6 Glycogen synthesis *in vitro*, with and without insulin, by rat diaphragms as a function of the phosphate concentration of the medium

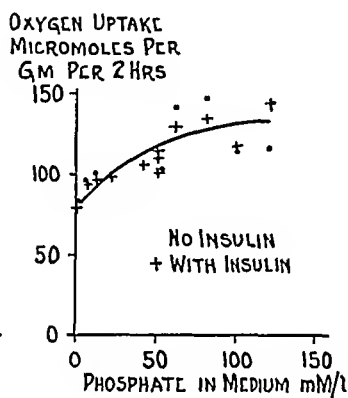


FIG 7

FIG 7 Oxygen uptake of rat diaphragms in glucose saline medium at varying concentrations of phosphate

at 0.300 M by the addition of NaCl. The glucose concentration was 0.3 per cent. In the complete absence of added phosphate in the medium (Fig 6) there is no significant diminution of the spontaneous glycogen synthesis, nor is the effect of insulin lessened. Up to 0.050 M, phosphate has no significant effect either upon spontaneous glycogen synthesis or the effect of insulin thereon. At higher concentrations glycogen synthesis is somewhat depressed.

There is, however, a definite effect of increasing phosphate concentration upon the oxygen uptake (Fig 7) which is almost doubled at the high phosphate. It is to be noted that insulin, although significantly increasing glycogen synthesis, has no effect upon the oxygen uptake. This was the uniform experience throughout a wide variety of conditions and is in complete conformity with the findings of Gemmell (1).

*Effect of Increasing Potassium Concentration upon Glycogen Synthesis*—Hastings (4) has discussed the significance of the intracellular ionic environment to enzymic activity of tissues *in vitro*. Since the intracellular cation is chiefly potassium, he sought to prevent undue losses of intracellular potassium from liver slices by using a suspending medium containing potassium rather than sodium. He found under these circumstances that glycogen synthesis from glucose was significantly greater than when the slices were suspended in a sodium medium. In view of this it was of interest to study the effect of variation in the relative proportions of sodium

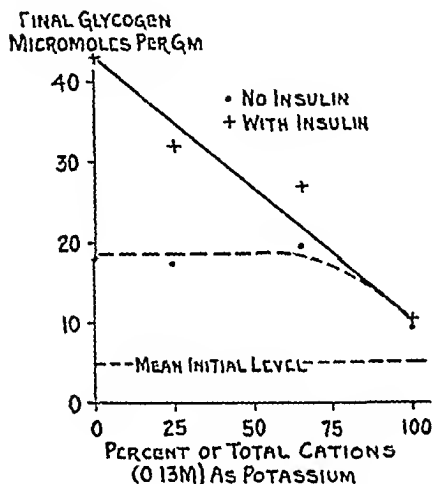


FIG 8

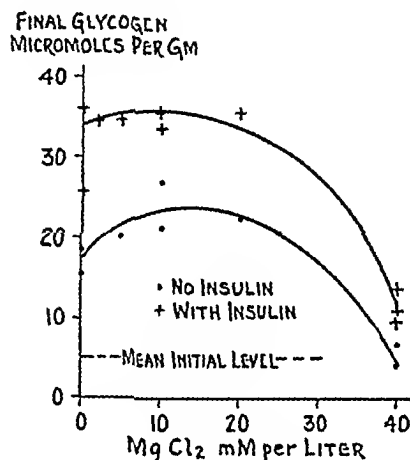


FIG 9

FIG 8 Glycogen synthesis *in vitro*, with and without insulin, by rat diaphragms as a function of increasing potassium concentration in the medium

FIG 9 Glycogen synthesis *in vitro*, with and without insulin, by rat diaphragms as a function of increasing magnesium concentration in the medium

and potassium in the suspending medium of the diaphragms during the synthesis of glycogen. The results of such experiments are shown in Fig 8.

It was found that the synthesis of glycogen without added insulin was uninfluenced by increasing the potassium up to 75 per cent of the total cations. At 100 per cent potassium glycogen synthesis was quite low. In the presence of insulin the results are striking. The maximum insulin effect was obtained in the complete absence of potassium. With an increasing proportion of potassium there was a linear decrease of the effect of insulin and at about 75 per cent potassium the insulin effect was completely abolished. It is obvious that ionic effects on metabolic reactions *in vitro* cannot be explained by any simple generalization. It is worth while noting, however, that the two different effects of potassium under

consideration were obtained under quite different conditions. Rat diaphragms are essentially in the intact state, whereas liver slices have large areas of cut surfaces. Permeability relations may readily be quite different and hence the two results obtained are not necessarily comparable.

**Effect of Varying Magnesium Concentration**—The effect upon the spontaneous glycogen synthesis and the insulin action (Fig 9) is approximately the same. In the absence of added magnesium the values tend to be lower than in the range 0.005 to 0.010 M. At higher concentration (0.040 M) glycogen synthesis without and with insulin is markedly reduced. Increasing concentrations of magnesium depress the oxygen uptake (Fig 10).

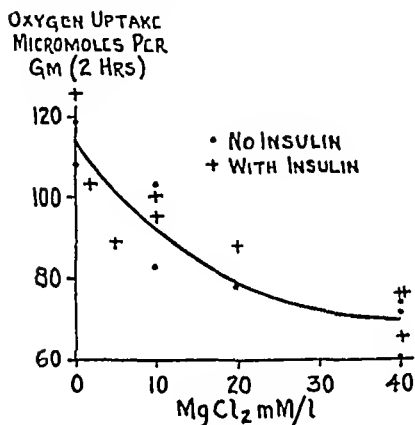


FIG 10

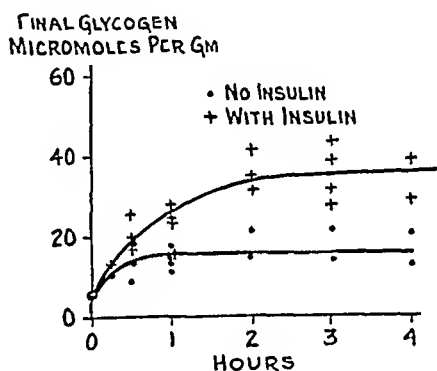


FIG 11

Fig 10 Oxygen uptake of rat diaphragms in glucose saline medium at varying concentrations of magnesium.

Fig 11 Glycogen synthesis *in vitro*, with and without insulin, by rat diaphragms as a function of time of equilibration.

Here again it is seen that insulin has no effect upon the total oxygen uptake, despite its marked action upon glycogen synthesis.

**Time Curve of Glycogen Synthesis**—Pairs of hemidiaphragms were equilibrated without and with insulin for periods of time varying from 15 minutes to 4 hours (Fig 11). Without insulin, glycogen synthesis reached a maximum in approximately 1 hour. With insulin, synthesis was more rapid, but leveled off at about 2 hours at approximately twice the value without insulin. The reason why glycogen does not go to still higher levels in the presence of insulin is a matter of interesting speculation. Diminution of metabolic activity *in vitro* cannot explain this, since the oxygen uptake of diaphragm *in vitro* continues unabated for periods up to 6 hours. Nor is it easy to assume that a high glycogen level by some mechanism prevents further synthesis, for it will be shown subsequently that

*Effect of Increasing Potassium Concentration upon Glycogen Synthesis*—Hastings (4) has discussed the significance of the intracellular ionic environment to enzymic activity of tissues *in vitro*. Since the intracellular cation is chiefly potassium, he sought to prevent undue losses of intracellular potassium from liver slices by using a suspending medium containing potassium rather than sodium. He found under these circumstances that glycogen synthesis from glucose was significantly greater than when the slices were suspended in a sodium medium. In view of this it was of interest to study the effect of variation in the relative proportions of sodium

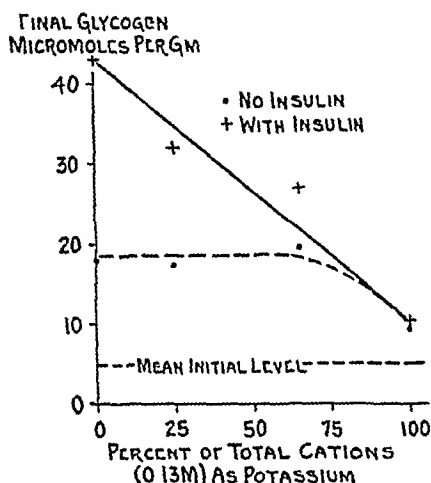


FIG 8

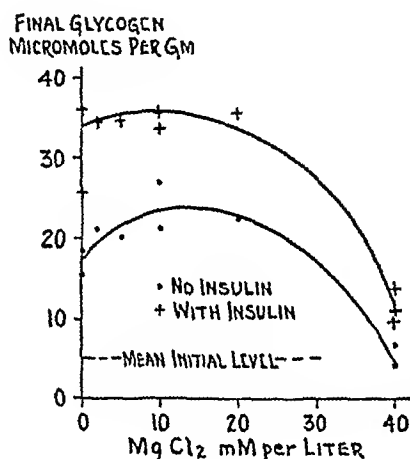


FIG 9

FIG 8 Glycogen synthesis *in vitro*, with and without insulin, by rat diaphragms as a function of increasing potassium concentration in the medium

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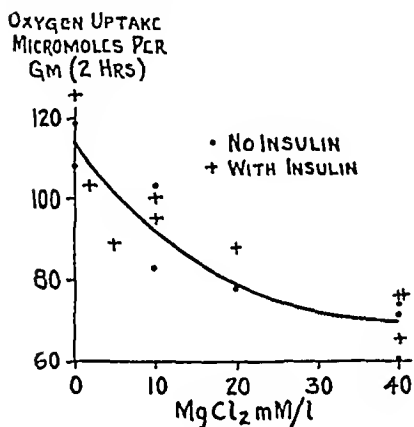


FIG 10

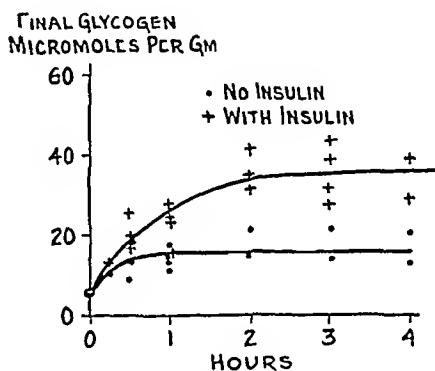


FIG 11

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under certain conditions glycogen synthesis by diaphragms *in vitro* can achieve levels of glycogen almost double the maximum value of 38 micromoles per gm attained in this experiment. Diminution of glucose concentration in the medium by reason of glycogen formation is insignificant and is, therefore, not a factor in the decrease of glycogen synthesis.

The data show that for most purposes an equilibration period of 2 hours is sufficient for maximum insulin effect.

*Insulin Effect in Diaphragms Containing High Initial Glycogen*—The experiments hitherto described were carried out on fasted normal rats. In consequence the initial glycogen of the diaphragm was quite low (mean

TABLE I  
*Glycogen Synthesis in Vitro by Diaphragms from Rats 4 Hours after Feeding Glucose by Gavage*

Rat No	Insulin	Oxygen uptake 0-1 hr	R Q	Glycogen		
				Initial	Final	Increase
	units per ml	micromoles per gm		micromoles per gm	micromoles per gm	micromoles per gm
II	0	59	0.87	51.5	58.9	7.4
IV	0	54	0.94	41.8	44.6	2.8
Mean		56	0.91	46.6	51.8	5.1
I	0.5	49	0.94	42.5	50.2	7.7
III	0.5	55	0.81	42.3	55.3	13.0
V	0.05	53	0.90	31.8	46.9	15.1
VI	0.05	106*	0.83	50.3	85.2	34.9
VII	0.005	101*	0.90	50.8	62.7	11.9
Mean		52	0.88	43.5	60.0	16.5

\* 2 hours

about 5 micromoles per gm). In the presence of 0.3 per cent glucose, the glycogen increased in 2 hours to a mean value of 20 micromoles per gm, while with glucose + insulin, the mean glycogen value was about 40 micromoles per gm. It was of interest to determine whether or not an initial high value of glycogen would limit the further synthesis without insulin or the effect of insulin in promoting extra glycogen formation. Rats were fed glucose by gavage. 4 hours later hemidiaphragms were equilibrated in 0.3 per cent glucose with or without insulin, the other hemidiaphragm being used to determine the initial glycogen value. The results are given in Table I.

The following points are to be noted (1) The initial glycogen was

somewhat higher than the final glycogen observed in the case of fasted rats (44 micromoles per gm = 0.78 per cent) (2) In the absence of insulin there was a relatively small increase of glycogen within 1 hour (3) The effect of insulin in bringing about an increase of glycogen was unimpaired by the initial high glycogen and was approximately that observed in the case of fasting rats with initial low glycogen (mean 18.5 micromoles = 0.34 per cent), and the final glycogen concentration was quite high (60 micromoles per gm = 1.1 per cent) (4) The average RQ (0.88) is significantly higher than that observed in fasted rats

#### DISCUSSION

It is obvious that the stimulating effect of insulin *in vitro* upon the synthesis of glycogen by rat diaphragm does not localize to any considerable degree the precise site of action of the hormone The effect could be readily explained by the recent experiments of Price, Cori, and Colowick (5) who, using tissue extracts, showed that insulin removes the existing inhibition of anterior pituitary hormone on the hexokinase reaction by which glucose is phosphorylated to glucose-6-phosphate In this connection, the study of the glycogen synthesis in hypophysectomized rats and rats injected with anterior pituitary extract would be most instructive to determine whether the findings would be consistent with the interpretation of the work of Price *et al* For if the action of insulin is *solely* to release the inhibitory action of anterior pituitary extract on the hexokinase reaction, no effect of insulin *in vitro* should be found when diaphragms from hypophysectomized rats are used and its action should be influenced by antecedent injection of anterior pituitary extract

Several questions are raised which remain unanswered (1) Why does the potassium ion have such a striking effect in decreasing the stimulating action of insulin in increasing glycogen synthesis? (2) Why does the diaphragm from a glucose-fed rat under the influence of insulin double its preexisting high level of glycogen in contrast to the diaphragm from a fasted rat, which stops synthesis at a much lower level despite apparently unimpaired metabolic activity?

The relation between the glucose concentration of the medium and the effect of insulin on glycogen synthesis is important in a consideration of theories of insulin action It is a conceivable hypothesis that insulin may have catalytic effects upon metabolic reaction involving glucose at relatively low concentrations of glucose, but that at higher concentrations of glucose insulin is dispensable, *i e*, reactions leading either to storage or oxidation would proceed independently of insulin This is equivalent to saying that insulin has no specific function in carbohydrate metabolism Apparently Hecter, Levine, and Soskin (6) advocated this hypothesis on t



basis of their experiments, among which are data on glycogen synthesis with rat diaphragms. They found that the synthesis of glycogen by rat diaphragm *in vitro* increased as the concentration of glucose was raised from 0.1 to 0.4 per cent. However, the effect of added insulin, which they found marked at the lower concentration of glucose, practically vanished at 0.4 per cent, indicating the dispensability of insulin at the high glucose concentration. Our experiments in two series of rats gave the

TABLE II

*Glycogen Synthesis and Organic Phosphate in Rat Diaphragms Equilibrated with Glucose with or without Insulin*

Rat No	Glucose in medium	Insulin in medium	Oxygen uptake	Final organic P	$\Delta$ organic P	Glucose utilization $\approx$ glycogen synthesis	$\Delta$ glucose utilization
	<i>per cent</i>		<i>micromoles per gm</i>	<i>micromoles per gm</i>	<i>micromoles per gm</i>	<i>micromoles per gm</i>	<i>micromoles per gm</i>
311	0.1	0	102	17.8		13.3	
		+	94	19.3	-1.5	31.8	17.5
312	0.1	0	208	27.7		24.3	
		+	186	15.4	-12.3	40.8	16.5
		0	169	18.3		23.2	
		+	164	17.0	-1.3	42.8	19.6
313	0.1	0	132	21.1		16.2	
		+	124	19.7	-1.4	32.4	16.2
316	0.3	0	151	33.4		33.1	
		+	152	50.2	+16.8	63.0	29.9
	0.5	0	157	36.9		60.4	
		+	137	43.4	+6.5	83.0	22.6
317	0.3	0	97	17.4		15.8	
		+	98	18.2	+0.8	52.4	36.6
	0.5	0	98	14.9		40.1	
		+	91	12.9	-2.0	40.2	0.1
Mean					+0.7		19.9 $\pm 4.2^*$

\* Standard error of the mean

opposite results. Rather than an elimination of the insulin effect, there was found a steadily increasing effect roughly proportional to the glucose concentration through the range 0.1 to 1.0 per cent. These results give no support to the above concept of insulin action.

Our experiments are in accord with those of Gemmill (1) on the striking lack of effect of insulin upon either the oxygen uptake or the RQ. In this connection it was observed in eight pairs of hemidiaphragms (Table II) that (1) insulin caused an extra synthesis of glycogen from glucose of about

20 micromoles per gm,<sup>1</sup> (2) the final organic phosphorus of the diaphragm was essentially the same with or without insulin, and (3) in the mean, there was no significant change of organic phosphorus during the period of glycogen synthesis. It is currently believed that glycogen synthesis requires a preliminary phosphorylation of glucose (hexokinase reaction) by adenosine triphosphate, and that adenosine triphosphate is only regenerated by oxidative reactions. This experiment shows that the extra adenosine triphosphate required for the extra synthesis of glycogen cannot come from that initially present in the diaphragm but must be synthesized by reaction directly or indirectly accelerated by insulin. How this extra activity can be achieved without some reflection in the total oxygen uptake or the R<sub>Q</sub> remains unexplained.

#### SUMMARY

1 The effect of insulin upon glycogen synthesis from glucose by rat diaphragm *in vitro* was studied with particular reference to the influence of variation of the following factors (a) glucose, insulin, phosphate, and magnesium concentrations in the medium, (b) pH of the medium, (c) initial level of glycogen in the diaphragm.

2 The time curve of glycogen synthesis by diaphragms and the effect of insulin upon organic phosphate were also studied.

3 The relation of the findings to theories of insulin action is discussed.

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<sup>1</sup> Since the diaphragm was needed for analysis of organic phosphate, the decrease of glucose in the medium was determined as a measure of glycogen synthesis. Control experiments showed this to be a sufficient approximation.



# THE EFFECT OF FLUOROACETATE ON ENZYMES AND ON TISSUE METABOLISM ITS USE FOR THE STUDY OF THE OXIDATIVE PATHWAY OF PYRUVATE METABOLISM\*

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(Received for publication, February 1, 1947)

Among the organic halogen compounds, those containing fluorine occupy a unique position regarding their chemical and physiological properties. Because of the large heat of formation of the C-F bond (114 kilocalories (1)), the addition of fluorine to the carbon atom brings forth, in most cases, a greater stability, especially among aliphatic compounds. This chemical stability is usually accompanied by lack of physiological action. Fluoroacetate is a striking exception to the physiologic inertia of fluorine aliphatic compounds, long ago it was found toxic to certain invertebrates, it was patented in 1927 (2) as a preservative against moths, and is now used as a rodenticide (3). This high toxicity of fluoroacetate compared with the non-toxicity of other organic aliphatic fluorine compounds is indeed of greater interest because the chemical stability is still maintained.

We present in this paper some of the results of work done in 1944 on the mechanism of action of fluoroacetate on enzymes and on the metabolism of animal tissues. The chemical stability of the fluorine of fluoroacetate and the lack of inhibition of the activity of isolated enzymes were in striking contrast to its powerful inhibition of acetate oxidation in animal tissues. Advantage has been taken of this inhibition to study the oxidative pathway of pyruvate metabolism.

## EXPERIMENTAL

The sodium fluoroacetate used in these experiments was prepared by hydrolysis of methyl fluoroacetate which was kindly provided by Professor M. S. Kharasch of the Department of Chemistry. The fluorine content of different samples varied from 0.1 to 1 per cent.

Most of the enzymes used to test the effect of fluoroacetate were prepared according to the methods summarized in Table I. Tyramine oxidase, choline oxidase, L-proline oxidase, and hydroxyacetic oxidase were the enzymes contained in suspensions of water-washed ground liver. Activity measure-

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

† With the technical assistance of Ruth Ahrens.

TABLE I  
Preparation of Enzyme Used for Testing Fluoroacetate Effects

Enzyme	Reacting substances	Substance determined	Method	Bibliographic reference No
Cytochrome oxidase	Reduced cytochrome <i>c</i> + cytochrome oxidase	Cytochrome <i>c</i> oxidation	Spectrophotometric	(4)
Succinoxidase	Succinate + cytochrome <i>c</i> + cytochrome oxidase	O <sub>2</sub> uptake	Manometric	(5)
Uricase	Uric acid	" "	"	*
D-Amino acid oxidase	D-Alanine + flavoprotein	" "	"	(6)
Lactic dehydrogenase	Lactate + DPN	DPN reduction	Spectrophotometric	(7)
Malic dehydrogenase	Malate + DPN	" "	"	(8)
Yeast alcohol dehydrogenase	Ethyl alcohol + DPN	" "	"	(9)
Catalase	H <sub>2</sub> O <sub>2</sub>	H <sub>2</sub> O	Titration	(10)
Yeast carboxylase	Pyruvate	CO <sub>2</sub> output	Manometric	(11)
Transaminase	$\alpha$ -Ketoglutarate + alanine	Glutamate formation	"	(12)
Enolase	3-Phosphoglycerate	Phosphopyruvate	Spectrophotometric	(13)
Xanthine oxidase	Xanthine + flavoprotein	O <sub>2</sub> uptake	Manometric	(14)
$\alpha$ -Ketoglutarate oxidase	$\alpha$ -Ketoglutarate	" "	"	(15)
Isocitric dehydrogenase	Isocitric acid + TPN + flavoprotein + methylene blue	" "	"	(16)
Glucose dehydrogenase	Glucose + DPN + cytochrome <i>c</i> + cytochrome oxidase	" "	"	(17)
Guanylic acid deaminase	Guanylic acid	NH <sub>3</sub> formation	Titration	(18)
Acid phosphatase	$\beta$ Glycerophosphate	H <sub>2</sub> PO <sub>4</sub> "	Spectrophotometric	(19)

DPN, diphosphopyridine nucleotide, TPN, triphosphopyridine nucleotide

\* Altman, K, and Barron, E S G, unpublished experiments



acid, lysine, tryptophan, glycylglycine, and alanine, fluoroacetate did not react at pH 7.4 in 24 hours

*Effect of Fluoroacetate on Isolated Enzymes*—The effect of fluoroacetate on three enzymes sensitive to the action of fluoride was tested, namely guanylic acid deaminase, acid phosphatase, and enolase. There was no effect from 0.01 M fluoroacetate on any of them.<sup>1</sup> At a concentration of 0.01 M, fluoroacetate had no effect on the activity of the following enzyme systems, all of them tested at their optimum activity: cytochrome oxidase, succinoxidase, choline oxidase, uricase, D-amino acid oxidase, lactate oxidase, malate oxidase, isocitric oxidase,  $\alpha$ -ketoglutarate oxidase, alcohol oxidase, yeast carboxylase, acetylcholinesterase, L-proline oxidase, catalase, transaminase, tyramine oxidase, hydroxyacetic oxidase, glucose oxidase, and xanthine oxidase. The oxidation of pyruvate by ground, washed pigeon liver suspensions was 18 per cent inhibited.

*Inhibition of Acetate Oxidation by Fluoroacetate*—The lack of action of fluoroacetate towards the enzyme systems mentioned above was in marked contrast with the inhibition of respiration of tissue slices. Sodium fluoroacetate (0.001 M to 0.01 M) produced appreciable inhibition on the  $O_2$  uptake. It must be pointed out that the respiration of guinea pig brain, which oxidizes acetate, was inhibited by 53 per cent, while that of rabbit, which does not oxidize acetate, was inhibited only by 11 per cent. This discrepancy between enzyme unreactivity and tissue respiration inhibition left one possibility to be tested, namely, that fluoroacetate might act as an inhibitor for the oxidation of acetate. This hypothesis was tested in experiments *in vitro* by addition of 0.005 M fluoroacetate to tissue slices 15 minutes before the addition of acetate and in experiments *in vivo* when fluoroacetate was injected into rats (5 mg per kilo). The fluoroacetate-treated rats were sacrificed when in moribund condition, and the rate of acetate utilization was measured and compared with that of similar tissues from normal rats (Table II). These experiments show that fluoroacetate is a powerful inhibitor of acetate metabolism.

*Effect of Fluoroacetate on Metabolism of Pyruvate*—In 1932, Barron and Miller (32) found that pyruvate was oxidized to acetate and  $CO_2$  by gonococci. Although the formation of acetate from pyruvate by animal tissue has also been reported (33–35), there is a tendency to ignore this oxidative pathway of pyruvate metabolism and to give as the main oxidative pathway that of pyruvate carboxylation to oxalacetate and condensation between pyruvate and oxalacetate as indicated by Kiebs in his "tricarboxylic acid

<sup>1</sup> Special care must be taken to free fluoroacetate from contaminating fluoride which might be formed during the preparation of this compound. Commercial preparations of fluoroacetate contain large amounts of fluoride and are unsuitable for enzyme experiments unless freed of fluoride.

eyele" (36) The powerful inhibiting effect of fluoroacetate on the oxidation of acetate can be used to test the oxidative pathway of pyruvate metabolism. If pyruvate is oxidized by tissues via Krebs' tricarboxylic acid cycle, there would be no accumulation of acetate and no inhibition of pyruvate metabolism, since fluoroacetate was found to have no effect on the oxidation of isocitric,  $\alpha$ -ketoglutaric, malic, and succinic acids by enzymes prepared from animal tissues. If, on the other hand, pyruvate is oxidized via the acetate pathway there would be an accumulation of acetate in the presence of fluoroacetate because of inhibition of acetate oxidation, and some inhibition of pyruvate oxidation, as consequence of this accumulation. Experiments to test these alternatives were performed with kidney, heart, and liver slices, and with muscle minced with the Latapie mincer.

TABLE II

*Effect of Fluoroacetate on Acetate Utilization by Heart, Kidney, and Liver Slices in Normal and Fluoroacetate-Treated Rats*

Acetate concentration, 0.014 M, fluoroacetate (in normal rats), 0.005 M, duration of experiments, 3 hours. Acetate utilization per mg of dry weight

Tissue	Control	Fluoroacetate	Inhibition
	c mm	c mm	per cent
Normal heart	7.6	0.8	90
Heart of poisoned rats	5.3		30
Normal kidney	25.3	9.5	62
Kidney of poisoned rats	18.2		28
Normal liver	10.4	4.3	59
Liver of poisoned rats	8.3		20

In rat kidney slices the inhibition produced by 0.01 M fluoroacetate on the  $O_2$  uptake was about the same in the absence or in the presence of pyruvate. There was an inhibition of pyruvate utilization and accumulation of acetate (Table III). In order to calculate the amount of pyruvate utilized by kidney slices by the acetate oxidative pathway it must be noted that 3.4 c mm of acetate found represent the acetate left unused as a consequence of fluoroacetate inhibition. Because this inhibition is about 70 per cent in the presence of 0.01 M fluoroacetate, the possible amount of acetate formed would be 4.9 c mm per mg per hour. Since the amount of pyruvate utilized in the presence of acetate was 11.1 c mm, it may be concluded that in kidney slices at least 44 per cent of the pyruvate was utilized via the oxidative pathway.

If succinate formation from pyruvate proceeds via oxidation to acetate, there must be inhibition of this process in the presence of fluoroacetate. On the other hand, if succinate is formed according to Krebs' tricarboxylic



acid cycle, fluoroacetate will have no effect on succinate formation, since none of the single reactions of the cycle are affected by it. In the presence of fluoroacetate (0.02 M) there was 77 per cent inhibition in the formation of succinate.

Some experiments were performed with human kidney obtained immediately after nephrectomy (hydronephrosis and sarcoma). The kidney slices used in the experiments had a normal appearance except perhaps those used in Determination I. The  $O_2$  uptake in the absence and in the presence of pyruvate, as well as the pyruvate utilization, was less inhibited by fluoroacetate (0.02 M) than in rat kidney slices. There was definite accumulation

TABLE III

*Effect of Fluoroacetate on Metabolism of Pyruvate by Kidney Slices*

Ringer-phosphate buffer, pH 7.4, pyruvate, 0.02 M, gas phase,  $O_2$ . Results per mg. of dry weight

Determination	Time	Fluoroacetate concentration	Control	Fluoroacetate	Inhibition
	hrs	M	c mm	c mm	per cent
$O_2$ uptake, no substrate	1	0.01	22.2	9.0	58
" " pyruvate	1	0.01	31.7	12.5	60.5
Pyruvate utilization	1	0.01	25.2	12.1	52
I Acetate formation, no substrate	2	0.02	None	1.01	Increase
Acetate formation, from pyruvate	2	0.02	1.01	5.4	"
Pyruvate utilization	2	0.02	38.2	26.9	29.5
II Acetate formation, from pyruvate	3	0.02	None	10.0	Increase
III Pyruvate utilization	2	0.02	29.2	17	42
Acetate formation	2	0.02	1.12	8.1	Increase
IV Pyruvate utilization	3	0.02	50.6	31.8	39
Acetate formation	2	0.02	1.35	7.9	Increase

of acetate in the presence of pyruvate and fluoroacetate. A good utilization of acetate by the kidney slices was largely inhibited on addition of 0.02 M fluoroacetate (Table IV).

Experiments with skeletal muscle were performed in minced muscle (Latapie) from pigeon breast and rabbit leg. In both cases the minced muscle was suspended in Krebs' phosphate-saline buffer free of Ca. Here, too, fluoroacetate inhibited the  $O_2$  uptake and pyruvate utilization. There was also accumulation of acetate (Table V). In rat heart slices there was also inhibition of  $O_2$  uptake and of pyruvate utilization.

The  $O_2$  uptake of rat liver slices was inhibited by 0.01 M fluoroacetate

There was a large inhibition of pyruvate utilization and small accumulation of volatile acid (no lanthanum test was performed to identify the acid) (Table V)

*Effect of Fluoroacetate on Fatty Acid Metabolism*—The inhibition produced by fluoroacetate in the  $O_2$  uptake of kidney slices whether in the presence or in the absence of fatty acids (acetate, acetoacetate, butyrate, crotonate) was about the same. There was, however, complete inhibition of the utilization of acetoacetate and about half inhibition of butyrate utilization (Table VI). Some inhibition of the  $O_2$  uptake was also found in rat liver slices, except with octanoate. There was inhibition of acetoacetate forma-

TABLE IV

*Effect of Fluoroacetate on Metabolism of Human Kidney (Slices)*

Ringer-phosphate buffer, pH 7.4,  $O_2$  as gas phase, substrates and fluoroacetate, 0.02 M. Results per mg of dry weight

Determination No	Time	Control	Fluoro acetate	Inhibition
	hrs	c mm	c mm	per cent
I $O_2$ uptake, no substrate	1	9.6	8.4	12.5
“ “ “ “	2	19.5	15.9	18
II “ “ “ “	1	13.2	9.9	25
“ “ “ “	2	24.5	18.5	24.5
I “ “ pyruvate	1	10.5	8.1	23
“ “ “ “	2	21.5	16.6	22
II “ “ “ “	1	15.1	10.3	32
“ “ “ “	2	29.7	20.8	30
I Pyruvate utilization	2	12.6	11.0	13
II “ “ “ “	2	18.2	15.2	16.5
III “ “ “ “	3	20.0	16.6	17
I Acetate formed from pyruvate	3	2.0	6.9	Increase
II “ “ “ “	3	4.6	14.3	“
I “ utilization	3	95.4	14.5	84.5
II “ “ “ “	3	11.2	1.1	91

tion from butyrate, caproate, octanoate, and valerate, with  $\beta$ -hydroxybutyrate and acetate, however, there was an increase (Table VII). Lehninger (37) found that the oxidation of acetate by minced muscle depended among other factors on the  $O_2$  tension. This effect has been made use of to distinguish the fluoroacetate inhibition. In liver slices, with air as the gas phase, 0.01 M fluoroacetate had no effect on the  $O_2$  uptake during the 1st hour whether in the presence or in the absence of acetate, when the gas phase was  $O_2$ , however, there was appreciable inhibition. These effects were more marked in the 2nd hour. Acetoacetate formation from acetate, greater in oxygen than in air, was increased to 190 per cent by fluoroacetate

in the presence of air and to 233 per cent in oxygen as gas phase (Table VIII)

*Effect of Fluoroacetate on Metabolism of Alanine, Lactate, and Glucose—* Since the oxidative pathway of the metabolism of pyruvate is inhibited by fluoroacetate, the effect of this inhibitor on the metabolism of alanine, lactate, and glucose in kidney slices was studied to provide a better under-

TABLE V

*Effect of Fluoroacetate on Metabolism of Pyruvate by Muscle, Liver, and Heart*

The experiments with heart tissue were of 2 hours duration. The results given were obtained with 5 cc of muscle suspensions containing 280 mg of fresh muscle

	O <sub>2</sub> uptake				Pyruvate utilization		Acetate formation	
	Without pyruvate		With pyruvate					
	Control	Fluoroacetate	Control	Fluoroacetate	Control	Fluoroacetate	Control	Fluoroacetate
Pigeon breast muscle								
	c mm	c mm	c mm	c mm	c mm	c mm	c mm	c mm
0.02 M fluoroacetate	1119.5	667.5	1982	1356	678	485	77.9	246.2
0.025 " "	1166	566.5	2060	1415	710	505		
0.01 " "	823.6	593.4	1803	1200	605	465	0	243
0.02 " "	962.5	420.6	1635.7	1060	715.4	354.3	0	394
Rabbit leg muscle								
	c mm	c mm	c mm	c mm	c mm	c mm	c mm	c mm
0.02 M fluoroacetate	495	122	646	441	504	380	163	238
0.02 " "	520	118	668	428	474	300	55	298
Rat liver								
	Q values	Q values	Q values	Q values	Q values	Q values	Q values	Q values
0.01 M fluoroacetate	8.6	5.2	8.7	4.6	10.2	2.7	0.04	0.13
Q values, 1st hr	7.8	3.3	9.7	2.4				
" " 2nd "								
Rat heart								
	Q values	Q values	Q values	Q values	Q values	Q values	Q values	Q values
0.01 M fluoroacetate	8.6	4.4	13.4	2.2	8.7	5.2		
Q values	10.4	7.8	13.2	3.4	7.8	6.3		

standing of the different pathways of pyruvate metabolism. The O<sub>2</sub> uptake was equally inhibited by fluoroacetate in the absence and in the presence of alanine. There was no effect on the formation of NH<sub>3</sub>. Pyruvate formation, extremely small in the control vessels, was increased 10 times in the presence of fluoroacetate. There was also slight accumulation of lactic acid in the tissues with fluoroacetate (Table IX). The accumula-

tion of pyruvate and the formation of lactate are undoubtedly due to a diminished removal by oxidation of the pyruvate formed or deamination of alanine

The inhibition of the  $O_2$  uptake in the presence of lactate was somewhat less than in the tissues not containing lactate. Lactate utilization was not affected (Table IX)

The  $O_2$  uptake in the absence of glucose was inhibited 55 per cent, while in the presence of glucose the inhibition was raised to 64 per cent. This increased inhibition was accompanied with an increase in the formation of lactic acid (aerobic glycolysis) and a decrease in the formation of pyruvic

TABLE VI

*Effect of Fluoroacetate on Fatty Acid Metabolism by Kidney Slices*

Fatty acid and fluoroacetate, 0.02 M Results per mg of dry weight

Determination No		Time	Control	Fluoroacetate	Inhibition
		hrs	c mm	c mm	per cent
I Acetate	$O_2$ uptake, no substrate	1	21.5	9.9	54
	" " acetate	1	27.1	9.1	66
					No acetoacetate accumulation
II Acetoacetate	$O_2$ uptake, no substrate	1	20.0	9.3	53.5
	" " acetoacetate	1	29.0	13.0	55
	Acetoacetate utilization	2	10.5	0	Complete
III Butyrate	$O_2$ uptake, no substrate	1	21.7	8.8	59.5
	" " butyrate	1	26.2	13.2	49.5
	Butyrate utilization	3	16.4	8.7	47.0
IV Crotonate	$O_2$ uptake, no substrate	1	21.7	8.8	59.5
	" " crotonate	1	24.7	11.3	54

acid. From the amount of lactic acid formed it can be seen that about half of the glucose metabolized was changed into lactic acid. The anaerobic utilization of glucose (glycolysis) was completely unaffected (Table X)

*Effect of Fluoroacetate on Synthesis of Carbohydrate by Kidney Slices*—It is known that kidney slices can synthesize carbohydrate from pyruvate (38). Although the mechanism of this synthesis is still unknown, it has been established that aerobic oxidative mechanisms are required because synthesis does not occur in the absence of oxygen. This synthesis was inhibited 82 per cent with 0.02 M fluoroacetate. The synthesis of carbohydrate from acetate was studied in kidney slices suspended in Ringer-bicarbonate solution containing 0.02 M acetate. In all experiments there

was an increase in carbohydrate in the samples containing acetate as compared with the samples without acetate. Fluoroacetate (0.01 M) inhibited this synthesis (Table XI).

TABLE VII

*Effect of Fluoroacetate on Fatty Acid Metabolism by Liver Slices*

Ringer-phosphate buffer, pH 7.4, substrates and fluoroacetate, 0.02 M. Results per mg of dry weight

Determination		Time	Control	Fluoroacetate	Inhibition
		hrs	c mm	c mm	per cent
Butyrate	O <sub>2</sub> uptake, no substrate	1	9.1	5.3	42
	" " butyrate	1	13.5	9.8	27
	Butyrate utilization	2	10.3	1.8	82
	Acetoacetate formation, no substrate	2	1.0	2.5	Increase
	Acetoacetate formation, from butyrate	2	9.2	3.6	61
	$\beta$ -Hydroxybutyrate formation, no substrate	2	0.8	0.8	None
	$\beta$ -Hydroxybutyrate formation, from butyrate	2	4.5	0.8	82
Caproate	O <sub>2</sub> uptake, no substrate	1	8.2	4.3	48
	" " caproate	1	12.9	7.0	46
	Caproate utilized	2	12.1	9.4	22
	Acetoacetate formation, from caproate	2	10.2	3.1	70
Octanoate	O <sub>2</sub> uptake, octanoate	1	13.5	11.7	13
	Acetoacetate formation	2	8.5	4.1	52
Valerate	O <sub>2</sub> uptake, valerate	1	14.1	8.5	40
	Acetoacetate formation	2	7.8	4.6	41
$\beta$ -Hydroxybutyrate	O <sub>2</sub> uptake, hydroxybutyrate	1	10.7	9.6	10
	Hydroxybutyrate utilized	1	5.4	5.6	None
	Acetoacetate formation	1	2.51	3.48	Increase
Acetate	O <sub>2</sub> uptake, acetate	1	12.1	7.0	42
	Acetoacetate formation, no substrate	2	2.6	3.9	Increase
	Acetoacetate formation, from acetate	2	4.2	5.9	"

*Effect of Fluoroacetate on Acetylations*—It has been postulated that the inhibition of acetate oxidation by fluoroacetate belongs to the type of substrate-competitive inhibitions. Fluoroacetate could be used to see whether acetate is the substance which combines with the protein moiety of the enzyme. The reaction would be inhibited by fluoroacetate if the

protein moiety combines with acetate. Three acetylations were studied the acetylation of sulfanilamide, of *p*-aminobenzoic acid by rabbit liver

TABLE VIII

*Effect of O<sub>2</sub> Tension on Fluoroacetate Inhibition of Respiration and Acetate Metabolism by Liver Slices (Rat)*

Acetate, 0.01 M, fluoroacetate, 0.01 M, Ringer-phosphate, pH 7.4. Results per mg of dry weight

Determination		Control	Fluoroacetate	Inhibition
		<i>c mm</i>	<i>c mm</i>	<i>per cent</i>
O <sub>2</sub> uptake in air	No substrate			
	1st hr	6.2	6.5	None
	2nd "	6.3	5.3	16
" " " O <sub>2</sub>	No substrate			
	1st hr	9.0	5.6	38
	2nd "	10.5	4.9	53
" " " air	With acetate			
	1st hr	6.4	6.1	None
	2nd "	6.4	5.8	12.5
" " " O	With acetate			
	1st hr	10.4	7.8	25
	2nd "	13.7	7.6	44.5
Acetoacetate formation	No substrate, in air	0.96	3.36	Increase 250
	Acetate " "	1.37	3.97	" 190
	No substrate, in oxygen	1.49	4.27	" 187
	Acetate, in oxygen	1.54	6.12	" 233

TABLE IX

*Effect of Fluoroacetate (0.01 M) on Metabolism of Alanine (DL-) and of Lactate by Rat Kidney Slices*

Determination	Time	Control	Fluoroacetate	Inhibition
	<i>hrs</i>	<i>c mm</i>	<i>c mm</i>	<i>per cent</i>
O <sub>2</sub> uptake, no substrate	1	23	10.7	53.5
" " alanine	1	30.4	13.8	54.5
NH <sub>3</sub> formation	2	8.5	8.9	None
Pyruvate formation	2	0.11	1.5	Increase
Lactate "	2	None	0.13	"
O uptake lactate	1	29.7	17.6	41
Lactate utilization	2	21.5	20.6	None

slices, and the acetylation of choline by chopped rat brain in the presence of glucose and of pyruvate. The acetylation of sulfanilamide and of *p*-aminobenzoic acid by rabbit liver slices was increased in the presence of

0.02 M fluoroacetate The acetylation of choline by chopped brain either in the presence of glucose or pyruvate was not affected by fluoroacetate (Table XII). The increase in acetylation observed in the experiments with sulfanilamide is undoubtedly due to the inhibition of acetate metabolism, thus providing more acetate for the acetylation process. It may be con-

TABLE X

*Effect of Fluoroacetate (0.02 M) on Metabolism of Glucose by Rat Kidney (Slices)*

Determination	Time	Control	Fluoroacetate	Inhibition
	hrs	counts	counts	per cent
O <sub>2</sub> uptake, no substrate	1	24.9	11.2	55
" " glucose	1	32.5	11.6	64
Glucose utilization	2.5	5.6	5.1	None
Lactate formation	2.5	2.9	5.4	Increase
Pyruvate "	2.5	0.3	0.16	47
Anaerobic glycolysis	1	7.6	7.5	None

TABLE XI

*Effect of Fluoroacetate on Synthesis of Carbohydrate from Acetate by Rat Kidney (Slices)\**

substrate, 0.01 M acetate, fluoroacetate, 0.01 M, incubation time 3 hours, temperature 38°, O<sub>2</sub>-CO<sub>2</sub> as gas phase, buffer, Ringer bicarbonate, pH 7.4

Experiment No	Carbohydrate in 100 mg tissue as c.m.m. glucose			
	No substrate	Acetate	Acetate + fluoroacetate	Inhibition
				per cent
1	36	105.5	56.6	70
2	59	76.3	68	48
3	62.4	78.8	68.7	61.5
4	80.6	96.0	66.5	Complete
5	79.2	93.4	88.1	37
6	80.6	108.5	104	16
7	72.4	84.2	70.1	Complete
8	80.0	94.6	73	"
9	48.7	80.8	66.2	45.5
Pyruvate as substrate	45.2	141	63.7	81

\* These experiments were performed by Mr. Ulric Presta

cluded from these experiments that in the acetylation of these three compounds it is the substance to be acetylated which combines with the protein moiety of the enzyme system and not the acetate, because the reaction is not affected by fluoroacetate.

*Effect of Fluoroacetate on Plant Growth*—Competitive inhibitors can be used to determine whether during the series of reactions which occur in the

utilization of foodstuffs or during synthesis there are obligatory pathways. Malonate inhibition of succinate oxidation has thus been used to determine whether in the stepwise degradation of carbohydrates and fats there is a step involving succinate oxidation. Similarly, fluoroacetate can be used to determine whether acetate oxidation is necessary in the process of growth of seeds. For these experiments three examples of seeds were taken with high fat content (tomato and cantaloupe), with high protein content (beans and peas), and with high carbohydrate content (corn and wheat). The seeds were grown in glazed earthenware pots on acid-washed quartz sand and distilled water with and without addition of fluoroacetate. The growth was followed for 3 weeks, at which time food reserves of the seeds approach depletion. With 0.01 M fluoroacetate, growth was almost completely inhibited in all the seeds. With tomato and pea seeds there was no growth. In a few cases, with the other seeds, the root tip just broke

TABLE XII

*Effect of Fluoroacetate (0.001 M) on Acetylations by Rabbit Liver (Slices)*

Sulfanilamide and *p*-aminobenzoic acid, 1 mg per vessel, buffer, Ringer-phosphate, pH 7.4, O<sub>2</sub> as gas phase, incubation time, 3 hours at 38°

Acetylation reaction	Acetyl compound formation per gm dry tissue		
	Control	Fluoroacetate	Increase
	<i>c mm</i>	<i>c mm</i>	<i>per cent</i>
Sulfanilamide, Determination I	672	920	37
“ “ II	784	1098	40
“ “ III	696	1010	60
<i>p</i> -Aminobenzoic acid, Determination I	705	1015	44
“ “ “ II	584	706	21
Choline	4	4	None

through the seed coat but no further development took place. This break through may have been due merely to a swelling of the seed on taking up water without any actual cell proliferation. With 0.001 M fluoroacetate there was marked inhibition of the growth of cantaloupe seeds but only a small effect on the growth of bean, pea, corn, and wheat seeds. Tomato seeds proved to be the most sensitive. With 0.001 M fluoroacetate the tomato seedlings apparently started, like the controls, for the first 3 days or until the root was 1 to 2 cm long. Further growth then stopped and the root gradually shriveled and browned. With 0.0003 M fluoroacetate there was still a significant inhibition of the growth of tomato seedlings.

#### DISCUSSION

The experiments presented in this paper have shown that fluoroacetate, a powerful toxic agent, lethal for small mammals at amounts varying from



0.5 to 5 mg per kilo, was completely ineffective against the activity of a large number of oxidative enzymes, although it inhibited the respiration of tissue slices. The experiments have shown furthermore that fluoroacetate is a powerful inhibitor of the oxidation of acetate by tissues not only when added *in vitro* but also when the oxidation of acetate was tested in the tissues of animals treated with fluoroacetate. This selective action, together with the studies of Kalnitsky and Barron (39), places fluoroacetate as an inhibitor belonging to the group of substrate-competitive inhibitors and is a strong indication in favor of the assumption that the inhibitions observed in tissue respiration are due to the existence of a phase of acetate oxidation in the series of reactions which in tissues result in the consumption of oxygen.

This striking inhibition of acetate oxidation by fluoroacetate has been utilized to test the oxidative pathway of pyruvate metabolism via acetate, an oxidation found to occur in bacteria but repeatedly denied to occur in all tissues. The experiments in which the metabolism of pyruvate was studied in the presence of fluoroacetate in kidney, liver, and muscle, have shown that there was accumulation of acetate and an inhibition of pyruvate utilization. Of the other pathways of pyruvate metabolism it has been shown that amination to alanine, reduction to lactate, and dismutation to lactate and acetate are not affected. Furthermore, none of the oxidative steps of the tricarboxylic acid cycle, oxidation of isocitric,  $\alpha$ -ketoglutaric, succinic, and malic acids, were affected by fluoroacetate.

It may therefore be concluded from these experiments that the toxicity of fluoroacetate is due to inhibition of the oxidative pathway of acetate metabolism. Fluoroacetate probably acts by inhibiting the formation of "active" acetate (the so called  $C_2$  compound, which may be an acetyl derivative or an acetate radical). The experiments with pyruvate have shown that oxidation of pyruvate to acetate and rapid disposal of the acetate thus formed occur in tissues. How much of the oxidative removal is performed via acetate and how much via carboxylation to oxalacetate has not been determined in all tissues. In kidney slices, at least 45 per cent of the pyruvate utilized could be accounted for as due to direct oxidation. Under the conditions of the experiment it is probable that because of acetate accumulation the metabolism of pyruvate deviated towards other pathways.

The experiments with fatty acids show an inhibition in the oxidation and utilization of these substrates with an inhibition of acetoacetate formation in liver slices. However, with acetate as substrate there was accumulation of acetoacetate. This inhibition of acetoacetate formation in the presence of fluoroacetate supports the view that the oxidation of fatty acids occurs by steps until the formation of acetate. In the metabolism of acetate there

are then two pathways oxidation, inhibited by fluoroacetate, and condensation to acetoacetate, not affected by it. The high toxicity of fluoroacetate is indication of the essential rôle which acetate metabolism plays in tissue metabolism.

#### SUMMARY

Fluoroacetate, which is highly toxic to animals, inhibited the oxidation of acetate by animal tissues both in experiments *in vitro* and in tissues of animals receiving lethal injections. Fluoroacetate had no effect on the activity of a large number of oxidation enzymes or fluoride-sensitive enzymes, nor did it combine with —SH groups. In the presence of pyruvate, fluoroacetate produced an accumulation of acetate accompanied by a diminished utilization of pyruvate. Since it had no effect on the other pathways of pyruvate metabolism or on the oxidative steps present in the tricarboxylic acid cycle it may be concluded that the direct oxidation of pyruvate to acetate is of great importance in animal tissues. Fluoroacetate inhibited the oxidation of fatty acids and produced an inhibition of acetoacetate formation. This is presented as evidence that fatty acid oxidation passes through an acetate step. Fluoroacetate had no effect on acetylation reactions such as the acetylation of sulfanilamide, *p*-aminobenzoic acid, and choline. Fluoroacetate inhibited the synthesis of carbohydrate from acetate or from pyruvate in kidney slices. Fluoroacetate was found to be toxic for the growth of seeds, especially seeds of tomato.

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# THE EFFECT OF FLUOROACETATE ON THE METABOLISM OF YEAST AND BACTERIA\*

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(Received for publication, February 1, 1947)

It was shown in the preceding paper (1) that fluoroacetate, which is extremely toxic to animals and plants, has a strong inhibitory effect on the metabolism of acetate. Because of the importance of acetate in intermediary metabolism, a more detailed study of this problem was undertaken, with biological material such as yeast and bacteria. Suspensions of bakers' yeast and *Corynebacterium creatinovorans* exhibit low endogenous activities and utilize acetate rapidly, two-thirds of the acetate is oxidized to carbon dioxide and water, and the remainder is converted to carbohydrate. Therefore, these two types of cells were used in attempts to elucidate the mechanism of action of fluoroacetate. The experiments presented in this paper show the great sensitivity of acetate metabolism by these organisms to fluoroacetate, the competitive nature of this inhibition, and the lack of inhibition in the presence of other related halogen compounds. Partial inhibitions are observed when other substrates are utilized in the presence of fluoroacetate. These can be attributed to the intermediate formation of acetate during the metabolism of these substrates, as is illustrated by the accumulation of acetate in several instances.

## Methods

The yeast used in these experiments was Fleischmann's bakers' yeast, which was washed by centrifugation three times with distilled water and kept in suspension in water. *Corynebacterium creatinovorans* was obtained from the American Type Culture Collection. The cell-free extracts of *Escherichia coli* were obtained by grinding the cells with Pyrex glass powder according to Kalnitsky, Utter, and Werkman (2), gonococci were grown in the egg digest medium of Miller and Castles (3).

Pyruvate was determined colorimetrically by the method of Friedemann and Haugen (4), acetate by double distillation according to Friedemann

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

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reversal of inhibition on the addition of higher concentrations of acetate (Table II)

*Effect of Fluoroacetate on Synthesis of Citrate from Acetate by Yeast*—It seems that citrate synthesis is due to a condensation of some form of acetate with oxalacetate (11-13), synthesis of citric acid would thus be the first step in the metabolism of activated acetate. Fluoroacetate produced a complete inhibition of the oxidation of Mg acetate and 90 per cent inhibition of citric acid formation (Table III). In Experiment III,

TABLE II

*Inhibition of Yeast Acetate Oxidation by Fluoroacetate, Partial Reversal on Addition of Acetate*

Acetate added 15 minutes after addition of fluoroacetate (0.001 M)

Acetate concentration	Inhibition	Reactivation
M	per cent	per cent
0.01	89.2	
0.04	62.4	
0.08	46.1	30.2
0.12	58.8	48.3
		34.2

TABLE III

*Effect of Fluoroacetate on Citrate Formation by Baker's Yeast*

Yeast cells, 8.5 mg, Mg acetate, 0.077 M, fluoroacetate, 0.005 M, duration of experiments, 4 hours. Blank values for oxygen uptake and citric acid subtracted

Experiment No	Citric acid formation		Inhibition	O <sub>2</sub> uptake	
	Control	Inhibitor		Control	Inhibitor
	c mm	c mm	per cent	c mm	c mm
I	50.0	4.5	91	1010	0
II	38.6	3.8	90	1005	0
III	51.0	14.0	72	1012	242

in which an old suspension of baker's yeast was used, the inhibition of O<sub>2</sub> uptake was lower (76 per cent), there was also a similar reduced inhibition of citric acid synthesis (72 per cent).

*Effect of Fluoroacetate on Oxidation of Ethyl Alcohol by Yeast*—During the oxidation of ethyl alcohol to acetate by baker's yeast, 1 mole of oxygen is taken up per mole of ethyl alcohol oxidized, according to the following equations

If fluoroacetate is a specific inhibitor of acetate oxidation, the  $O_2$  uptake of yeast in the presence of ethyl alcohol will not be influenced by fluoroacetate, at least for some time because the rate of oxidation of ethyl alcohol is much faster than that of acetate. This was found to be the case (Fig 2). The rate of  $O_2$  consumption was the same in the absence and in the presence of fluoroacetate until 670 c mm of  $O_2$  had been taken up. From then on the degree of inhibition increased rapidly. At the end of the experiment, the control vessel containing ethyl alcohol had used 1572 c mm of  $O_2$  and produced 150 c mm of acetate, while, in the presence of

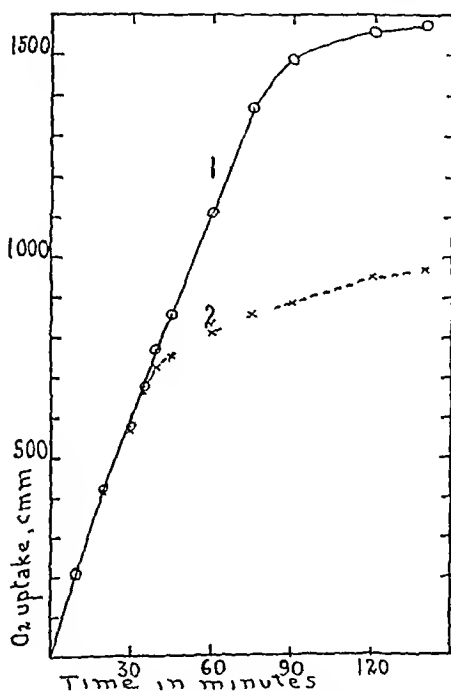


FIG 2 Effect of fluoroacetate (0.01 M) on the oxidation of ethyl alcohol by bakers' yeast. Curve 1, ethyl alcohol, Curve 2, ethyl alcohol + fluoroacetate

fluoroacetate plus ethyl alcohol, 975 c mm of  $O_2$  were consumed and 890 c mm of acetate were formed. The amount of ethanol added was 940 c mm. In the presence of fluoroacetate, then, there was approximately a ratio of alcohol- $O_2$ -acetate of 1:1:1 as required in equations (1) and (2).

*Effect of Fluoroacetate on Formation of Pyruvate and Acetate from Glucose*—The  $O_2$  uptake of bakers' yeast as well as the formation of  $CO_2$  in the presence of glucose was only slightly affected by fluoroacetate during the first 2 hours. At the end of 120 minutes there appeared a definite inhibition of the  $O_2$  uptake and of  $CO_2$  formation (Table IV). The inhibition

became apparent when a change in the rate of  $O_2$  uptake and  $CO_2$  evolution took place. The sudden change in the rate of  $O_2$  uptake might be an indication of the completion of one series of reactions and the beginning of another, this latter series being the one inhibited by fluoroacetate. That such may be the case was shown in experiments which were carried out with 5 cc of the same yeast suspension plus phosphate buffer plus 1.2 mm of glucose in a total volume of 21 cc. At the end of 2 hours, when the rate

TABLE IV

*Effect of Fluoroacetate on Oxidation of Glucose by Bakers' Yeast*

Yeast (5 per cent suspension), 0.5 cc, phosphate buffer, pH 6.44, 0.033 M, glucose, 0.01 M, fluoroacetate, 0.0033 M

Time	$O_2$ uptake		Inhibition	$CO$ evolution		Inhibition
	Glucose	Glucose + fluoroacetate		Glucose	Glucose + fluoroacetate	
min	cc mm	cc mm	per cent	cc mm	cc mm	per cent
0-60	-567	-501	11.6	953	870	8.7
60-120	-603	-580	3.8	456	387	15.1
120-150	-179	-116	35.3	97	44	54.7
150-180	-171	-73	57.4	127	70	45.0
180-210	-125	-72	42.4	100	69	31.0
0-210	-1645	-1342	18.4	1733	1440	16.9

TABLE V

*Effect of Fluoroacetate on Formation of Pyruvate and Acetate by Bakers' Yeast*

Phosphate buffer, 0.03 M, pH 6.44, glucose, 0.03 M, fluoroacetate, 0.0083 M, temperature,  $38^\circ$ , 10 per cent yeast suspension, 1.5 cc for 9 cc total volume, duration of experiments, 3 hours

Substrate	Pyruvate formation		Acetate formation	
	Control	Fluoroacetate	Control	Fluoroacetate
	cc mm	cc mm	cc mm	cc mm
No substrate	5.38	17.4	20.2	12.7
Glucose	235	148	712	1732

of  $O_2$  uptake had changed in the manometric experiments, 82 per cent of the glucose originally present had disappeared, at the same time there was formation of 0.22 mm of volatile acid. At the end of 3 hours, there were left 0.03 mm of glucose and 0.101 mm of volatile acid. Therefore, during the 3rd hour, 0.119 mm of volatile acid was utilized. The inhibition of  $O_2$  uptake by fluoroacetate observed during the 3rd hour was due to inhibition of oxidation of the acetic acid accumulated, as is demonstrated in the experiments of Table V. The formation of acetate increased in the

presence of fluoroacetate by 140 per cent. The acetic acid was further identified by the lanthanum test of Kluge and Tschich (14).

*Effect of Fluoroacetate on Oxidations Produced by Bakers' Yeast*—The effect of fluoroacetate (0.003 M) on a number of oxidations produced by yeast is given in Table VI. Inhibition of pyruvate oxidation was the only one which approached the extent of acetate inhibition. However, with bakers' yeast washed twice with distilled water, suspended in water, and shaken for 24 hours in the presence of oxygen, it was possible to demonstrate in experiments of 1 hour's duration that, while acetate oxidation was completely inhibited by 0.00075 M fluoroacetate, the oxidation of pyruvate was only 79 per cent inhibited. The inhibition of  $O_2$  uptake with pyruvate was accompanied by accumulation of volatile acid. Yeast

TABLE VI

*Effect of Fluoroacetate on Oxidations Produced by Bakers' Yeast*

Substrate concentration, 0.02 M, fluoroacetate, 0.003 M, gas phase, air, temperature, 37°, duration of experiment, 1 hour

Substrate	pH	$O_2$ uptake		Inhibition
		Control	Fluoroacetate	
		<i>c mm</i>	<i>c mm</i>	<i>per cent</i>
None	6.2	82	40	51
Glucose	6.2	567	501	11.6
Lactate	7.0	150	75	50
Pyruvate	4.0	105	16	85
Ethyl alcohol	7.0	650	595	8.5
Acetate	7.0	160	0	Complete
Citrate	Water	37	22	40.5
Malate	"	62	38	38.7
Succinate	"	82	41	50

(60 mg, dry weight, in 21 cc of fluid) suspended in hippurate buffer (0.01 M, pH 4.0) and pyruvate (0.5 mM) produced only 0.2 micromole of volatile acid in 1 hour, while in the presence of 0.003 M fluoroacetate 12.25 micromoles were produced. This inhibition, as previously postulated, can be presented as evidence for the reversibility of the oxidative reaction.

Further proof that fluoroacetate inhibits only the initial step of acetate metabolism is given in the experiments plotted in Fig. 3, in which the  $O_2$  uptake of yeast in the presence of acetate was followed for 120 minutes. In the first 30 minutes, fluoroacetate produced an inhibition of 85 per cent in the  $O_2$  uptake. From this moment on, the  $O_2$  uptake of the samples containing fluoroacetate started to rise and at the end of 90 minutes it was proceeding at the same rate as the controls. This release of inhibition with



time can be explained in the light of the previous experiments  $O_2$  uptake started as soon as there was enough citric acid formed, and oxidation of carboxylic acid proceeded unaffected by fluoroacetate

*Effect of Fluoroacetate on Bacterial Metabolism*—The inhibition of acetate metabolism by fluoroacetate shown in animal tissues and yeast has also been observed in bacteria

The oxidation of acetate by *Corynebacterium creatinovorans* was completely inhibited by 0.002 M fluoroacetate. The inhibition of  $O_2$  uptake

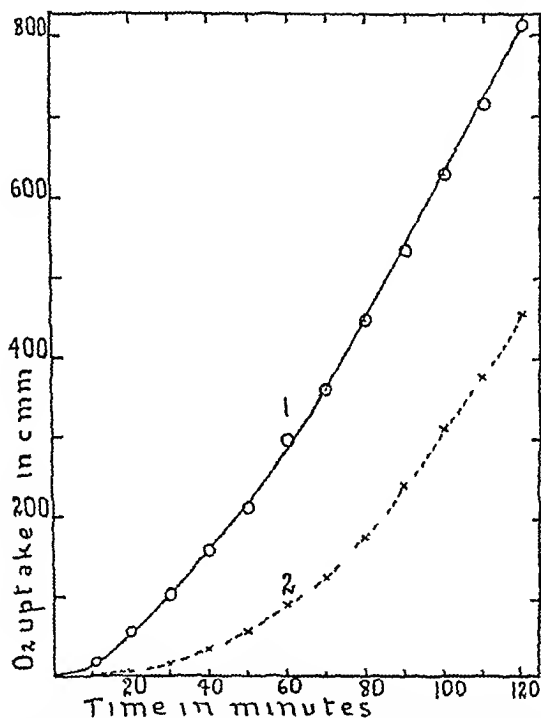


FIG 3 Release of fluoroacetate inhibition with time. Fluoroacetate concentration, 0.0004 M, acetate concentration, 0.01 M, Curve 1, control, Curve 2, fluoroacetate

was accompanied by complete inhibition of acetate utilization and of carbohydrate synthesis (Table VII)

The effects of fluoroacetate and fluorobutyrate on the endogenous respiration of these bacteria were quite different from the effects obtained with animal tissues and yeast cells. Both inhibited the respiration of ground kidney cortex, 38 to 43 per cent. Fluoroacetate inhibited the endogenous respiration of bakers' yeast to the same extent, whereas fluorobutyrate showed no inhibitory action. The endogenous respiration of *Corynebacterium creatinovorans* was not only uninhibited by fluoroacetate

and fluorobutyrate but definitely increased (Table VIII). The mechanism of this increase is not known. It is possible that these fluoro acids divert cellular metabolism towards the oxidative pathways in the same manner as do low concentrations of dinitrophenol, cyanide, and azide (15). In Table IX data are given on the effect of fluoroacetate and fluorobutyrate on oxidations produced by *Corynebacterium creatinovorans*. While fluoroacetate inhibited the oxidation of acetate completely, fluorobutyrate had no effect at all. In general, fluorobutyrate inhibition was smaller or non-existent. Fluoroacetate had strong inhibitory power on the  $O_2$  uptake in

TABLE VII  
Effect of Fluoroacetate on Acetate Metabolism by  
*Corynebacterium creatinovorans*

Experiment	Control	Fluoroacetate	Inhibition
	<i>c mm</i>	<i>c mm</i>	<i>per cent</i>
$O_2$ uptake, no substrate	271	305	None
" " acetate	2061	298	Complete
Acetate utilization	1340	0	"
Carbohydrate formation from acetate	148	0	"

TABLE VIII  
Effect of Fluoroacetate and Fluorobutyrate on Endogenous Respiration  
of Tissues and Isolated Cells

Concentration of fluoro fatty acids, 0.002 M

Tissue	$O_2$ uptake		
	Control	Fluoroacetate	Fluorobutyrate
	<i>c mm</i>	<i>c mm</i>	<i>c mm</i>
Rabbit kidney (ground)	148	92	85
Bakers' yeast	31	18	30
<i>Corynebacterium creatinovorans</i>	101	131	139

the presence of malate, glycolate, glyoxalate, glycine, and succinate, substances which are very slowly oxidized by *Corynebacterium creatinovorans*.

Succinate is oxidized by *Corynebacterium creatinovorans* very slowly and the  $QO_2$  succinate is only 2.5. However, when the cells are frozen quickly and dried *in vacuo* and then resuspended in water, they oxidize dicarboxylic acids quite rapidly.<sup>1</sup> Fluoroacetate (0.002 M) inhibited 76 per cent the  $O_2$  uptake by these dried bacteria in the presence of succinate. Malonate, however, produced only 11 per cent inhibition (Table X). Whether this lack of malonate inhibition is due to non-penetration through the cell membrane is not known.

<sup>1</sup> Barron, E. S. G., and Singer, T. P., to be published.

TABLE IX

*Effect of Fluoroacetate and Fluorobutyrate on Some Oxidations Produced by Corynebacterium creatinovorans*

Substrate	Inhibition		Substrate	Inhibition	
	Fluoroacetate	Fluorobutyrate		Fluoroacetate	Fluorobutyrate
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>
None	None	None	Glycerol	18	5
Acetate	Complete	"	Malate	97	None
Glucose	70	20	Glycolate	Complete	
Pyruvate	68	None	Glyoxalate	86	
Lactate	65	13	Glycine	87	
Butyrate	61	4	Succinate (vacuum dried cells)	83	

TABLE X

*Effect of Fluoroacetate and Malonate on Oxidation of Succinate by Dried Cells of Corynebacterium creatinovorans*

Cells, 10 mg, dry weight, phosphate buffer, 0.04 M, pH 7.0, succinate, 0.02 M, malonate, 0.033 M, fluoroacetate, 0.002 M, temperature, 38°, duration of experiment, 1 hour

	O <sub>2</sub> uptake	Inhibition
	<i>c mm</i>	<i>per cent</i>
No substrate	119	
" " + fluoroacetate	119	None
Succinate	238	
" + fluoroacetate	147	76
Malonate	119	
Succinate + malonate	225	11

TABLE XI

*Effect of Fluoroacetate on Metabolism of Pyruvate by Escherichia coli and Gonococci*

Temperature, 38°, duration of experiments, 1 hour

		Control	Fluoroacetate	Inhibition
		<i>c mm</i>	<i>c mm</i>	<i>per cent</i>
<i>E. coli</i>	Pyruvate, O <sub>2</sub> uptake	262	125	52
	" CO <sub>2</sub> output (N <sub>2</sub> -CO <sub>2</sub> as gas phase)	48	50	None
" " cell-free preparation	Pyruvate, CO <sub>2</sub> output (N CO as gas phase)	310	298	"
	Acetyl phosphate formation	289	287	"
Gonococci	Pyruvate, O <sub>2</sub> uptake	170	102	40
	" + acetate	126	61	51.6

*Effect of Fluoroacetate on Pyruvate Oxidation*—The experiments with animal tissues and with yeast have shown that fluoroacetate exerts a definite inhibition of pyruvate metabolism in animal tissues and a more powerful effect in yeast. To study the mechanism of this inhibition further two species of bacteria have been used *Escherichia coli*, which oxidizes pyruvate and acetate and dissimilates pyruvate anaerobically to acetic acid and formic acid, and gonococci, which oxidizes pyruvate to acetate. The oxidation of pyruvate by *E. coli* was inhibited 52 per cent by fluoroacetate (0.003 M). Anaerobically, in bicarbonate buffer, with  $N_2-CO_2$  as the gas phase, fluoroacetate had no effect on the  $CO_2$  output, even when the fluoroacetate concentration was increased to 0.01 M. With a cell-free extract of *E. coli* in the presence of bicarbonate and phosphate buffer (pH 6.88) there was no inhibition of the dissimilation reaction with 0.01 M fluoroacetate. Since acetyl phosphate has been shown to be an intermediary in this reaction (16), determinations of this compound were made both in the presence and in the absence of fluoroacetate (0.01 M). There was no effect on acetyl phosphate formation. The oxidation of pyruvate by gonococci was inhibited 40 per cent by 0.01 M fluoroacetate. Pyruvate oxidation by these bacteria was also inhibited 26 per cent by addition of 0.02 M acetate. In the presence of 0.02 M acetate, fluoroacetate inhibition rose to 52 per cent (Table XI). These experiments favor the view that fluoroacetate inhibition of pyruvate oxidation in bacteria is due to accumulation of acetate.

#### DISCUSSION

The inhibition of acetate metabolism by fluoroacetate when added to yeast and bacterial suspensions 15 minutes before acetate addition, the complete lack of inhibition when fluoroacetate is added after the addition of acetate, and the partial reversal of the inhibition on addition of increasing quantities of acetate, are evidence that this is a competitive inhibition in which the inhibitor and the substrate compete for combination with the protein moiety of the enzyme:  $\text{protein} + \text{acetate} \rightleftharpoons \text{protein-acetate}$ ,  $\text{protein} + \text{fluoroacetate} \rightleftharpoons \text{protein-fluoroacetate}$ .

The lack of inhibition shown by the other halogen acetates, chloro-, bromo-, iodo-, and fluoro fatty acids other than acetic, and finally the lack of action of trifluoroacetate, demonstrate the remarkable specificity of monofluoroacetate. This specificity seems to be due to the close geometric resemblance to acetate, since the difference between the atomic distances of the C-H bond and the C-F bond is only of 0.32 Å. This specificity would be in agreement with the lock and key resemblance of the combination between substrate and protein, i.e., the space group on the side chains of the protein molecule where acetate is anchored must

be arranged in such a manner that only molecules very closely resembling acetic acid can find anchor in the space of those side chains. This specificity was most clearly seen when the rate of oxidation of ethanol by bakers' yeast was followed, because the rate of oxidation of ethanol to acetate is faster than that of acetate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ .

The inhibition of citric acid synthesis from acetate and the lack of action of fluoroacetate on the oxidation of isocitrate,  $\alpha$ -ketoglutarate, succinate, and malate by their respective oxidizing enzymes are offered as evidence that the inhibition of acetate metabolism by fluoroacetate takes place in the first step of the series of reactions which end in the formation of  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , *i.e.*, the activation of acetate, necessary for the condensation process which ends in citric acid formation, is a process which according to Lynen (12) continues through the oxidative steps of the tricarboxylic acid cycle.

The inhibition of pyruvate metabolism seems to be limited to the oxidative step. In gonococci, in which pyruvate oxidation ends in the formation of acetate +  $\text{CO}_2$ , the inhibition produced by fluoroacetate was less than half of the inhibition produced on the metabolism of acetate by yeast and *Corynebacterium creatinovorans*. Furthermore some inhibition was observed on addition of acetate, an indication that fluoroacetate inhibition may be due to the accumulation of acetate. On the other hand, the inhibition produced by fluoroacetate on the oxidation of pyruvate by bakers' yeast almost approached that of acetate. This powerful inhibition could be interpreted as being due to direct action of fluoroacetate on the activating protein of pyruvic oxidase.

In the experiments with *Corynebacterium creatinovorans* the inhibition of glycolic acid ( $\text{CH}_2\text{OHCOOH}$ ), of glyoxalic acid ( $\text{CHOCOOH}$ ), and of glycine ( $\text{CH}_2\text{NH}_2\text{COOH}$ ) oxidation can be considered as resembling the inhibition of acetic acid oxidation, *i.e.*, competitive inhibition, because the chemical structure and size of these molecules resemble those of  $\text{CH}_3\text{FCOOH}$ . The strong inhibition of malate and succinate oxidation by fluoroacetate was in marked contrast with the lack of action on the isolated enzymes extracted from animal tissues. Since the pathways of malate and succinate oxidation in these bacteria are not known, we refrain from conjectures. Suffice it to recall that Slade and Werkman (17) have given evidence that *Acrobacter indologenes* can split succinate into acetate and that Kalnitsky *et al* (18) have demonstrated the formation of succinate from acetate by a cell-free preparation of *Escherichia coli*.

#### SUMMARY

The oxidation of acetate by bakers' yeast was completely inhibited when monofluoroacetate was added to yeast 10 minutes previous to the

addition of acetate. When acetate was added before monofluoroacetate, there was no inhibition, an indication that fluoroacetate acts as a competitive inhibitor. The inhibition was partially released on addition of more acetate. This inhibitory power is restricted to monofluoroacetate, for none of the other halogen acetates, nor trifluoroacetate, nor higher fluoro fatty acids had any effect. The inhibition seems to occur in the first step of acetate metabolism, as is shown by the inhibition of citric acid synthesis from acetate and the accumulation of acetate during the oxidation of glucose and of ethanol by bakers' yeast and by the eventual release of the inhibition.

The inhibition of pyruvate oxidation by fluoroacetate was strong in bakers' yeast and not so marked in gonococci, *Escherichia coli*, and *Corynebacterium creatinovorans*.

The oxidation of acetate, glycolate, glyoxalate, glycine, malate, and succinate by *Corynebacterium creatinovorans*, as well as the synthesis of carbohydrate from acetate, was strongly inhibited by monofluoroacetate. The possible mechanism of these inhibitions is discussed.

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# EFFECT OF TEMPERATURE ON FADING OF THE CARR-PRICE COLORS OF VITAMIN A AND COMMON CAROTENOID PIGMENTS\*

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(Received for publication, June 5, 1947)

The transient character of the blue color produced by the interaction of vitamin A with a chloroform solution of antimony trichloride has been recognized since the introduction of the reagent by Carr and Price (1), and has been considered to be a more or less serious fault inherent in the Carr-Price method for the estimation of vitamin A. However, since the introduction of the modern photoelectric photometers, by means of which absorption measurements may be made within a few seconds of the color development, the rapid fading of the color has become less objectionable. Indeed, Oser *et al* (2) and Brew and Scott (3) have utilized the rapidity of the fading of the vitamin A color developed by the Carr-Price reagent to differentiate it from the similar colors produced by the reagent with the carotenoid pigments which frequently accompany the vitamin in biological extracts. Meunier and Raoul (4) have suggested similar methods to distinguish vitamins A<sub>1</sub> and A<sub>2</sub>.

Two physical factors, light and temperature, are known to influence the rate of fading of the Carr-Price colors. The effect of light on the fading of the color due to the natural esters of vitamin A has been investigated by Caldwell and Parrish (5). An extension of this work to other forms of vitamin A and to the common carotenoids<sup>1</sup> has indicated a much greater sensitivity of the vitamin A Carr-Price color to intense illumination than is observed with the carotenoids. Brew and Scott (3) make use of the destructive effect of light in their technique for the estimation of vitamin A in the presence of large amounts of mixed carotenoids.

Although several investigators have noted the effect of variation in temperature on the development and fading of the Carr-Price colors with vitamin A, in no case did the study extend to the various forms of the vitamin or to the related carotenoid pigments. Wokes and Willmott (6), using the Lovibond tintometer, studied the fading of the Carr-Price colors with cod liver oil. Their results are substantiated by those reported here with pure vitamin A. Dann and Evelyn (7) have reported variations in

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<sup>1</sup> Caldwell, M. J., and Hughes, J. S., in preparation



the intensity of color with temperature Norris and Church (8), making similar observations, have recommended the use of a constant temperature room in which to make the Carr-Price measurements It appears likely from the data presented here that these early observations of less color at higher temperatures are the result of the increased fading rate at high temperatures rather than of a difference in the initial color

### *Procedure*

*Materials*—The Carr-Price reagent was prepared according to Koehn and Sherman (9) The antimony trichloride was dissolved in the purified chloroform at the rate of 22.5 gm per 100 ml of solvent The reagent was stored at room temperature in subdued light and filtered before use

Vitamin A as the crystalline alcohol, crystalline acetate, and as the liquid concentrate of the natural esters was used in the investigation Prior to use, these were preserved in the dark at  $-20^{\circ}$  Stock solutions in U S P chloroform were prepared, and from these suitable dilutions for the Carr-Price study were made

The seven most common carotenoid pigments listed by Zechmeister (10) were available These were  $\alpha$ -carotene,  $\beta$ -carotene,  $\gamma$ -carotene, lycopene, cryptoxanthin, lutein, and zeaxanthin With the exception of  $\gamma$ -carotene, these were obtained from other investigators or from commercial sources The  $\gamma$ -carotene was prepared from the orange-colored jelly-like mass exuded from the tilial galls of the common juniper during cool, damp weather Smits and Peterson (11) tentatively identified the yellow pigment in this mass as  $\gamma$ -carotene by means of a Bausch and Lomb visual spectrophotometer Using an extraction and purification technique similar to theirs, except that heat and alkali were avoided to minimize isomerization, the authors obtained a concentrated extract having an absorption spectrum (Beckman) corresponding closely to that of the partially isomerized  $\gamma$ -carotene reported by Zechmeister (10)

All of the pigments were stored at  $-20^{\circ}$  until used Solutions in U S P chloroform of approximately 100  $\gamma$  per ml were prepared for the Carr-Price study The concentration and purity were established in all cases by reference to the spectral data found in the literature (10, 12)

*Apparatus and Methods*—For this study a thermally controlled photometer was constructed This instrument was similar to the Evelyn photometer (13), except that the cell holder was a double walled water jacket, having a window at the proper height to allow the passage of light from the lamp through the absorbing solution to the photocell The absorption cells (Evelyn tubes), 620 m $\mu$  filter, photoelectric cell, and reflecting galvanometer were the same as those supplied with the commercial Evelyn photometer (Rubicon) Auxiliary equipment used in connection with the

colorimeter consisted of a constant temperature water bath, centrifugal pump to circulate water through the jacket, and a special water-jacketed inner ball valve pipette for dispensing the Carr-Price reagent at any chosen temperature

The preliminary calibration of the photometer to 100 per cent transmission was made with a mixture of 1.0 ml of chloroform and 9.0 ml of reagent, the instrument and mixture being held at the desired temperature. An Evelyn tube was then placed in position, and 1.0 ml of the chromogenic solution under test was transferred to the tube and allowed to reach thermal

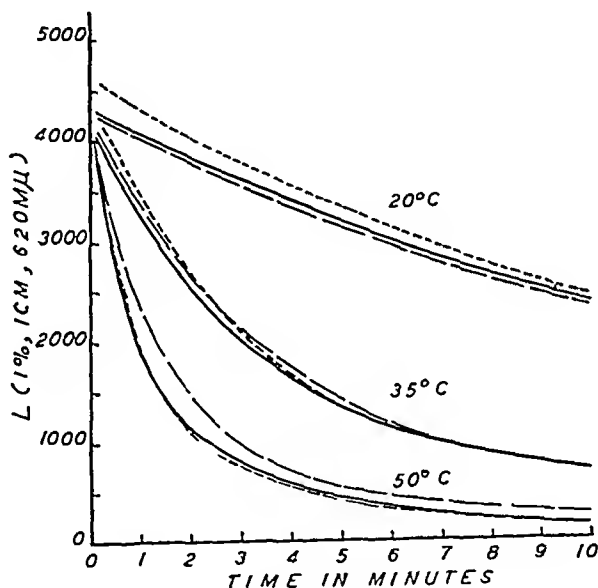


FIG 1 Effect of temperature on the fading of the blue color of the Carr-Price reaction mixture with vitamin A alcohol (solid line), vitamin A acetate (dash line), and vitamin A concentrate of the natural esters (dotted line). The  $L$  values are in terms of the alcohol equivalent.

equilibrium. Then 9.0 ml of the reagent at the same temperature were added rapidly from the special pipette, and absorption readings were taken periodically, starting 6 seconds after mixing. Duplicate determinations were made in all cases. Fading rates at temperatures of 20°, 35°, and 50° were determined for each of the chromogens.

**Calculations**—The photometric data were obtained in terms of percentage transmission. These data were converted to the linear function  $L_{1\text{cm}}^{1\%}$  by the relation,

$$L_{1\text{cm}}^{1\%} = \frac{2 - \log G}{c \cdot l}$$

where  $G$  is the galvanometer reading or percentage transmission,  $c$  is the concentration of the chromogen in the blue solution in per cent, and  $l$  is the thickness of the absorbing layer in centimeters. This term corresponds to the  $E_{1\text{cm}}^{1\%}$  commonly employed in spectrophotometric work

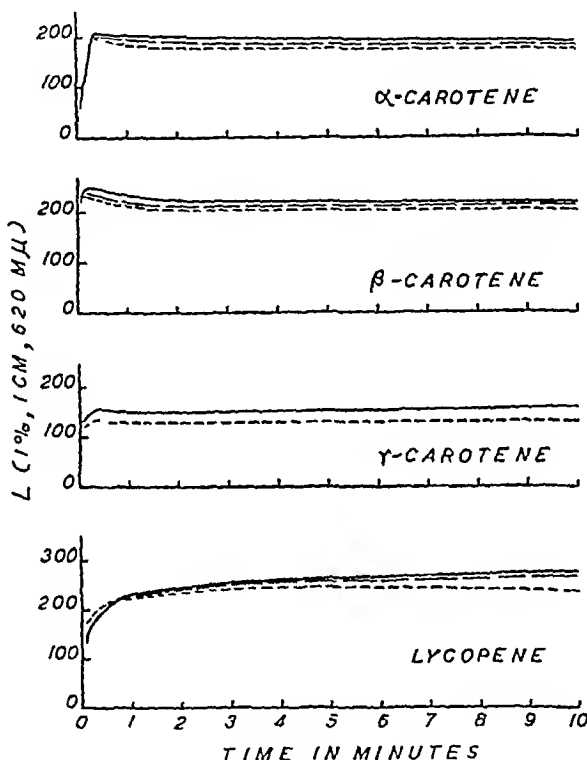


FIG 2 Effect of temperature on the fading of the blue color of the Carr-Price reaction mixtures with the hydrocarbon carotenoid pigments,  $\alpha$ -carotene,  $\beta$  carotene,  $\gamma$ -carotene, and lycopene, at 20° (solid line), 35° (dash line), and 50° (dotted line)

#### DISCUSSION

The results of this study are presented graphically in Figs 1, 2, and 3. In these figures  $L_{1\text{cm}}^{1\%}$  at 620  $m\mu$  values are plotted against time after mixing. In Fig 1 the alcohol, acetate, and natural ester forms of vitamin A are plotted together for comparison. In Figs 2 and 3 the curves represent the fading curves obtained with the carotenoid-antimony trichloride complexes at temperatures of 20°, 35°, and 50° respectively. It should be recognized that the  $L_{1\text{cm}}^{1\%}$  values and the fading curve obtained with a filter colorimeter or with a grating instrument with equally wide wave band are to a degree a function of the instrument used in the measurement (14)

Similar, although not identical, curves would be obtained with other instruments

The curves in Fig 1 reveal no major differences in the intensity or in the rate of fading of the Carr-Price colors of the various forms of vitamin A. By extrapolating the curves to zero time, it appears that the maximum color is developed almost instantaneously, and is independent of temperature. The rapidity of full color development in the case of vitamin A is

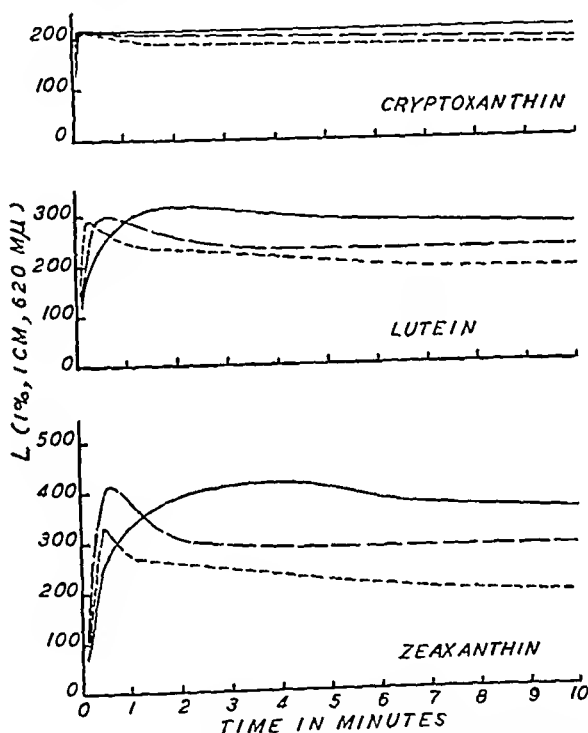


FIG 3 Effect of temperature on the fading of the blue color of the Carr-Price reaction mixtures with the carotenols, cryptoxanthin, lutein, and zeaxanthin, at 20° (solid line), 35° (dash line), and 50° (dotted line)

also indicated by the work of Gibson and Taylor (15) and Caldwell and Hughes (16) who used the "dynamic method" involving the use of a steady state of flowing reactants. Marked differences in the fading rates observed at the different temperatures are evident, and it is probable that this accounts for the early observations of less color at higher temperatures. From the data presented, it seems probable that normal variations in room temperature would be insufficient to affect seriously the Carr-Price determination, provided that the absorption measurements were made within a few

seconds of the mixing of the reactants. A logarithmic plot of the data indicates a first order reaction only at the lower temperature, with a shift to higher orders as the temperature rises.

In Fig. 2 are presented the fading rate curves of the hydrocarbon carotenoids,  $\alpha$ -carotene,  $\beta$ -carotene,  $\gamma$ -carotene, and lycopene. These curves are characterized by a color development period ranging from a few seconds, in the case of  $\beta$ -carotene, to several minutes in the case of lycopene, followed by a decrease to a plateau value. Both the color-producing and color-destroying reactions appear to be slightly accelerated by the higher temperatures.

In Fig. 3 are shown the corresponding curves for the carotenols, cryptoxanthin, lutein, and zeaxanthin. The cryptoxanthin curves closely resemble those of the other vitamin A-active carotenoids. Lutein and zeaxanthin, however, are characterized by a slow development of color, strongly influenced by temperature. At the higher temperatures the rise to maximum color is followed by an abrupt drop to the plateau values.

The difficulties encountered in the Carr-Price determination of vitamin A in the presence of high concentrations of the carotenoids is clearly indicated by a study of Figs. 1, 2, and 3. The absorption measurements are usually made within 10 seconds of the mixing of the reactants. Unfortunately this timing is coincidental with the rapidly increasing portion of the color curves for several of the interfering chromogens. Under these conditions high precision is not to be anticipated.

Within the range studied, the maximum intensity of color due to the vitamin A is developed before any measurement can be taken, regardless of the temperature. The rate of color development in the carotenoid-antimony trichloride reaction mixture is suppressed by low temperatures. It would therefore appear feasible, by special low temperature techniques, to suppress the carotenoid interference and make possible a satisfactory determination of vitamin A in the presence of large amounts of the common carotenoid pigments.

#### SUMMARY

1. Curves of the rate of fading at temperatures from 20–50° for the Carr-Price colors of three forms of vitamin A and the seven most common carotenoid pigments have been presented.

2. The maximum Carr-Price color of vitamin A in any of the forms studied is produced with extreme rapidity, and appears to be independent of the temperature.

3. The rate of fading of the vitamin A Carr-Price color is strongly accelerated by an increase in temperature.

4. Color development and destruction in the Carr-Price reaction mixture

with the carotenoids is much slower than with vitamin A. Both processes are accelerated by higher temperatures.

5 A possible method for the determination of vitamin A in the presence of large amounts of the carotenoids by utilizing the differences in the kinetics of the reactions is suggested.

The authors wish to thank Dr L Zechmeister, Dr John Porter, Dr G Mackinney, and Dr P L Harris for their contributions of the carotenoid and vitamin A preparations used in this study. Further, we wish to acknowledge the technical assistance of Mrs Howard Hamlin who aided materially in the project.

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# THE RIBONUCLEASE ACTIVITY OF NORMAL AND PARASITIZED CHICK ERYTHROCYTES\*

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(Received for publication, April 25, 1947)

The breakdown of yeast ribose nucleic acid by crystalline ribonuclease and tissue preparations has been studied by numerous investigators (1-8). However, there are few data on the nucleic acid metabolism of microorganisms. MacFadyen (9) reported that *Bacillus subtilis* can degrade yeast nucleic acid, and Woodward (10) studied the ribonuclease activity of *Pasteurella pestis*. No studies on the nucleic acid metabolism of malarial parasites have been reported.

The development of asexual forms of the malarial parasite in the red blood corpuscle is a rapid process. This is associated with the synthesis of relatively large amounts of nuclear material, which can be readily demonstrated by standard staining techniques. The malarial parasite must therefore possess enzymes which can synthesize, and, presumably, degrade nucleic acids.

At the present time, malarial parasites cannot be cultivated *in vitro*, free of the red cell. It is most convenient, therefore, to compare the normal and parasitized red blood cell in order to determine the activity of the parasite or the changes induced in the normal cell by the parasitization process. The study reported here deals with some aspects of the breakdown of yeast nucleic acid by normal chick red blood cells, and by red cells parasitized by *Plasmodium gallinaceum*.

## Methods

Chicks weighing 50 to 75 gm were infected with *Plasmodium gallinaceum*. Sufficient blood inoculum was used to parasitize 70 to 90 per cent of all red cells in 7 to 10 days. The animals were killed by severing the jugular vein. Blood from normal and parasitized chicks was heparinized by collection in a flask which contained 1 to 2 mg of heparin for 25 cc of blood. After erythrocyte and parasite counts had been made, the

\* The opinions and conclusions contained in this paper are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Navy Department.

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blood was centrifuged at high speed, the red cells were washed once with physiological saline solution, hemolyzed with approximately 3 mg of saponin per billion red blood cells, and washed three times with physiological saline. The residual preparation, which consisted largely of cell nuclei or cell nuclei and parasites, was diluted to contain the equivalent of 1 to 3 billion red blood cells per cc.

Yeast nucleic acid<sup>1</sup> was purified by precipitation with 5 volumes of glacial acetic acid (4). The precipitate was washed five times, by centrifugation, with a mixture of 5 volumes of glacial acetic acid and 1 volume of water (10), it was finally washed with alcohol, then ether, and was dried in an. Fresh solutions of nucleic acid for experiments were prepared daily. weighed quantities of nucleic acid were suspended in water and were dissolved by the careful addition of concentrated  $\text{NH}_4\text{OH}$  to the desired pH, usually 7.0.

For certain experiments on the rate of inorganic phosphorus formation, nucleic acid was hydrolyzed by autoclaving with  $\text{NH}_4\text{OH}$  (11), it was treated once with charcoal and was then neutralized to pH 7.0. Even after treatment with charcoal, the solution had a dark brown color. This preparation, which will be referred to as "nucleic acid hydrolysate," contains both mononucleotides and tetranucleotides.

Incubations were carried out in Warburg vessels at  $38^\circ$ , usually for 1 hour. The atmosphere was air, unless otherwise specified, and the volume was 3 to 5 cc. Veronal acetate buffer (12) or bicarbonate buffer, pH 7.4, with the proper gas mixture (95 per cent  $\text{O}_2$ -5 per cent  $\text{CO}_2$ , or 95 per cent  $\text{N}_2$ -5 per cent  $\text{CO}_2$ ), was used. After the desired period of incubation, the contents of the vessels were precipitated by the addition of an equal volume of 20 per cent  $\text{CCl}_3\text{COOH}$  or 0.5 per cent uranium acetate in 10 per cent  $\text{CCl}_3\text{COOH}$  (9). The filtrates were analyzed for soluble phosphorus.

These two precipitants were used, since they appear to fractionate, roughly, two different products in the breakdown of nucleic acid. Since uranium acetate precipitates, almost quantitatively, all molecules larger than nucleotides (9), the phosphorus content of uranium acetate filtrates must represent nucleotide and inorganic phosphorus. The amount of organic phosphorus found in 20 per cent  $\text{CCl}_3\text{COOH}$  filtrates is considerably higher than that in uranium acetate filtrates. It appears, therefore, that filtrates from solutions precipitated with 20 per cent  $\text{CCl}_3\text{COOH}$  contain molecules larger than nucleotides. It is reasonable to assume that the additional organic phosphorus in  $\text{CCl}_3\text{COOH}$  filtrates represents nucleic acid which has been merely depolymerized, probably to the tetranucleotide stage. Further hydrolysis produces the nucleotide, which is soluble in uranium acetate solution.

<sup>1</sup> Obtained from the Eastman Kodak Company or The Fleischmann Company.

In all experiments a nucleic acid blank solution was prepared and incubated in exactly the same manner as the experimental solutions. The blood preparation was added to the blank vessel after the addition of the precipitant. The precipitated mixtures were filtered through Whatman No. 42 filter paper. The addition of a filter aid, Filter-Cel,<sup>2</sup> made it possible to obtain clear filtrates after the addition of 20 per cent  $\text{CCl}_3\text{COOH}$ , although in some instances repeated filtrations were necessary. 1 cc of filtrate was digested with 1 cc of perchloric acid. The digestion mixture was diluted to a suitable volume, usually 50 cc, and the phosphorus content of an aliquot was determined by the method of Gomori (13). Inorganic phosphorus was determined directly on the filtrate by the same method. In some experiments, the addition of molybdic acid for the estimation of inorganic phosphorus caused the formation of a yellow precipitate. This could be filtered off readily, and did not interfere with the estimation of inorganic phosphate.

### Results

*Comparison of Normal and Parasitized Hemolyzed Red Blood Corpuscles*—Both normal and parasitized chick red blood cells possess enzymes which degrade nucleic acid. Enzymes of normal red cells can catalyze the first reaction, the depolymerization, in which  $\text{CCl}_3\text{COOH}$ -soluble phosphorus is formed, but they produce comparatively little of the second fraction, the lower molecular weight, uranium acetate-soluble nucleotide. Normal red cells liberate no inorganic phosphorus from nucleic acid. On the other hand, parasitized cells contain enzymes which catalyze all three reactions, they produce high concentrations of both organic phosphorus fractions, and they liberate inorganic phosphorus. Parasitized cells form almost twice as much tetranucleotide phosphorus, and 4 to 5 times as much nucleotide phosphorus, as a corresponding number of hemolyzed normal cells. The amount of inorganic phosphorus formed by parasitized cells is small but significant. Some typical experiments are listed in Table I.

Since the hydrolysis of nucleic acid is accompanied by the liberation of phosphoric acid groups, there is an increase in acidity. This can be measured manometrically as carbon dioxide production by conducting experiments in bicarbonate buffer, pH 7.4, in an atmosphere of 95 per cent  $\text{N}_2$ -5 per cent  $\text{CO}_2$ . Manometric studies reveal a marked difference in the rate of acid production from nucleic acid by hemolyzed normal and hemolyzed parasitized cells (Fig. 1). Parasitized cells always show a spontaneous acid production in the absence of added substrate, which is greater than that shown by normal cells. The addition of 200 mg of nucleic acid in a total volume of 3 cc caused a slight inhibition of the spontaneous

<sup>2</sup> Johns-Manville

acid production by normal cells, and a marked stimulation of acid production by parasitized cells. Since chemical estimations reveal that hemolyzed normal cells can depolymerize nucleic acid but cannot hydrolyze it to inorganic phosphate, the manometric results suggest that the phosphoric acid hydroxyl group liberated in depolymerization cannot be detected in a solution which has the strong buffer capacity of nucleic acid. The manometric data are roughly an index of the rate of complete hydrolysis to inorganic phosphate.

Manometric studies also reveal that washed parasitized corpuscles do not hydrolyze nucleic acid to inorganic phosphate before saponin hydrolysis (Fig 2), since the addition of nucleic acid to washed cells before hemolysis does not increase the rate of  $\text{CO}_2$  evolution.

TABLE I  
*Decomposition of Ribose Nucleic Acid, Comparison of Hemolyzed Normal and Parasitized Cells*

Experiment No	Cells	No of cells per vessel $\times 10^6$	Per cent parasitized	$\text{CCl}_3\text{COOH}$ soluble P	Uranium acetate soluble P	Inorganic P
				$\gamma$	$\gamma$	$\gamma$
1	Normal	2	0	1385	80	0
	Parasitized		85	2830	460	21.7
2	Normal	2.6	0	570	120	0
	Parasitized		60	1465	450	11.0
3	Normal	3.5	0	672	98	
	Parasitized		71	2392	549	
4	Normal	1.4	0	1120		
	Parasitized		67	2140		
5	Normal	1.4	0	1260		
	Parasitized		83	1880		

Total volume 4 cc, 100 mg of nucleic acid per vessel, veronal acetate buffer, pH 7.0. Incubated 1 hour at  $38^\circ$ .

When, instead of nucleic acid, nucleic acid which has been partially hydrolyzed by  $\text{NH}_4\text{OH}$  is used as substrate, inorganic phosphorus and free acid are rapidly liberated by hemolyzed parasitized cells. On the other hand, preparations of normal cells have practically no effect on this substrate (Fig 3). Most of the phosphorus in  $\text{NH}_4\text{OH}$ -hydrolyzed nucleic acid is soluble in  $\text{CCl}_3\text{COOH}$ . It has been found that the rate of production of both inorganic phosphate and free acid is a function of the concentration of  $\text{CCl}_3\text{COOH}$ -soluble phosphorus in the reaction vessel: acid production, measured manometrically, reaches its maximum at a concentration of approximately 5 mg of soluble phosphorus per 3 cc; inorganic phosphorus does not reach a maximum value even at a concentration of 10 mg of soluble phosphorus per 3 cc.

**Substrate Concentration**—The optimum substrate concentration for normal and parasitized hemolyzed erythrocytes was determined. Different quantities of nucleic acid were incubated with the blood preparation for 1 hour at 38°, and the concentration of soluble phosphorus was estimated after precipitation with the uranium acetate reagent. Since the maximum phosphorus production occurs at a concentration of about 200 mg of nucleic acid in 3 cc (Table II), the optimum substrate concentration is approximately 0.03 M.

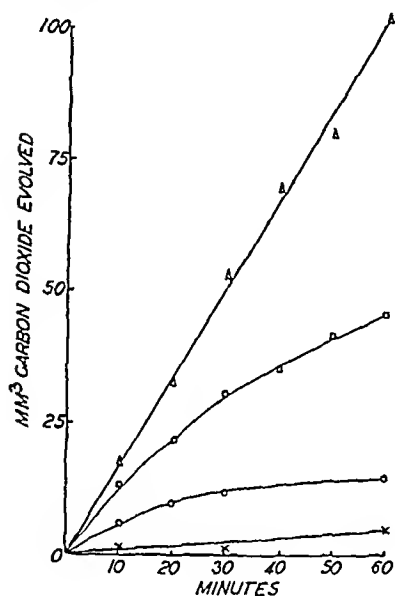


FIG 1

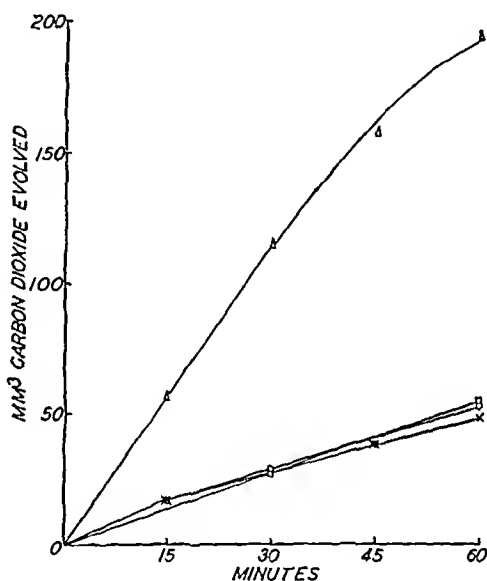


FIG 2

FIG 1 Rate of acid production from ribose nucleic acid by hemolyzed normal and parasitized corpuscles. O, normal control, X, normal + nucleic acid, □, parasitized control, Δ, parasitized + nucleic acid.

FIG 2 Effects of hemolysis on the rate of acid production from ribose nucleic acid. O, washed, parasitized erythrocytes, □, washed, parasitized erythrocytes + nucleic acid, X, hemolyzed parasitized erythrocyte, Δ, hemolyzed parasitized erythrocytes + nucleic acid.

**Optimum pH**—Material obtained from normal and parasitized cells following hemolysis by saponin was incubated with 100 mg of nucleic acid in a total volume of 4 cc for 1 hour at 38°. Veronal acetate buffer was used to provide the desired pH. The exact pH was determined by glass electrode on solutions prepared in exactly the same manner as the experimental ones. Soluble phosphorus was determined after precipitation with uranium acetate or  $\text{CCl}_3\text{COOH}$ .

The optimum activity for both normal and parasitized cells was near

pH 7.0 It was essentially the same for both the uranium acetate and  $\text{CCl}_3\text{COOH}$  fraction (Fig. 4)

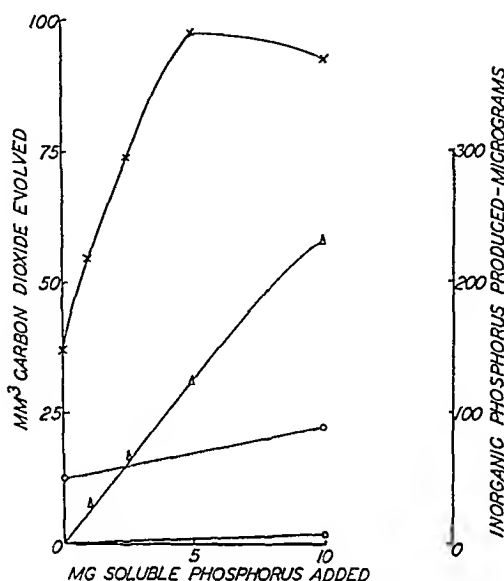


FIG. 3 Acid produced and inorganic phosphorus formed from nucleic acid hydrolysate in 1 hour O,  $\text{CO}_2$  formation (equivalent to acid) by hemolyzed normal blood, X,  $\text{CO}_2$  formation by hemolyzed parasitized blood, □, inorganic phosphorus-hemolyzed normal blood, Δ, inorganic phosphorus-hemolyzed parasitized blood

TABLE II  
*Optimum Substrate Concentration*

Nucleic acid per 3 cc	Uranium acetate soluble phosphorus	
	Normal	Parasitized*
mg	γ	γ
50	127	311
100	167	456
200	188	576
300	110	560

\* 60 per cent Each vessel contained the equivalent of  $1.7 \times 10^9$  red blood cells Bicarbonate buffer, pH 7.4, 95 per cent O 5 per cent  $\text{CO}_2$  Incubated 1 hour at  $38^\circ$

*Effect of Heat*—Since it has been reported that ribonuclease from other sources is unusually heat-stable (1, 3, 10), we have studied the ribonuclease activity of red cells after exposure to high temperature. Suspensions of hemolyzed normal cells and hemolyzed parasitized cells were diluted with saline to contain the same number of red cells per unit of volume,

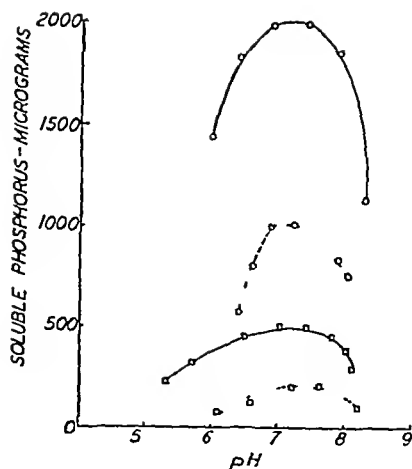


FIG 4 Effect of pH on the formation of uranium acetate and trichloroacetic acid-soluble phosphorus from ribose nucleic acid by normal and parasitized, hemolyzed corpuscles O, trichloroacetic acid-soluble P, parasitized cells, O—, trichloroacetic acid-soluble P, normal cells, □, uranium acetate soluble P, parasitized cells, □—, uranium acetate soluble P, normal cells

TABLE III

*Effect of Exposure to High Temperature on Ribose Nucleic Acid Breakdown by Normal and Parasitized Erythrocytes*

Experiment No	Cells	Cells per vessel $\times 10^6$	Per cent parasitized	Time of exposure at 100	Uranium acetate soluble P		CCl <sub>3</sub> COOH soluble P	
				min	$\gamma$	per cent inhibition	$\gamma$	per cent inhibition
1	Normal	2 0	0	0	72		640	
2	“	2 0	0	20	0	100	430	33
				5	44	67		
				20	4	97		
3	Parasitized	2 0	86	0	460		1210	
				5	50	89	520	57
				10	60	87	560	54
				20	30	93	510	58
4	Normal	3 5	0	0	120		570	
				20	40	67	390	32
	Parasitized			60	0	450		1465
5	Normal	3 5	0	20	42	90	545	63
				0	88		672	
				5	23	74	453	33
	Parasitized		71	20	22	75	372	44
				0	949		2392	
				5	113	88	852	64
		20	113	88	552	77		

Incubated 1 hour at 38°, 100 mg of nucleic acid per vessel, veronal acetate buffer pH 7.0

they were heated, unbuffered, in a boiling water bath for 5 minutes or 20 minutes. The coagulum so produced was homogenized in a ground glass tube, and its activity was compared with that of the controls.

Heating at 100° for 5 and 20 minutes caused 67 to 100 per cent inhibition of the production of uranium acetate-soluble phosphorus by both normal and parasitized cells (Table III). However, the formation of  $\text{CCl}_3\text{COOH}$ -soluble phosphorus was inhibited only 30 to 40 per cent for normal cells, and 54 to 77 per cent for parasitized cells.

TABLE IV

*Effect of Quinacrine and Quinine on Decomposition of Ribose Nucleic Acid by Parasitized Red Blood Cells*

Experiment No	Experiment	Cells per vessel $\times 10^6$	Percent parasitization	Uranium acetate soluble P	$\text{CCl}_3\text{COOH}$ -soluble P	Inorganic P
				$\gamma$	$\gamma$	$\gamma$
1	Control	2.1	74	480	1850	
	$10^{-3}$ M quinine			470	1450	
	$10^{-3}$ " quinacrine			485	1800	
	$5 \times 10^{-4}$ M quinine				2225	
	$5 \times 10^{-4}$ " quinacrine				2000	
2	Control	2.0	72	405	1720	21.4
	$10^{-3}$ M quinine			415	1472	16.1
	$10^{-3}$ " quinacrine			415	1600	20.2
3	Control	1.2	70		1780	49.0
	$5 \times 10^{-4}$ M quinine				2100	47.3
	$5 \times 10^{-4}$ " quinacrine				2000	46.0
4	Control	1.6	78		1750	
	$5 \times 10^{-4}$ M quinacrine				1750	
5	Control	1.7	56		1720	
	$10^{-3}$ M quinine				1472	
	$10^{-3}$ " quinacrine				1200	

Incubation time, 1 hour at 38°. Buffer, veronal acetate, pH 7.4. 100 mg of nucleic acid per vessel.

*Effect of Inhibitors*—The action of quinine and quinacrine on the ribonuclease activity of hemolyzed parasitized cells was studied, since both compounds are effective antimalarial drugs. No significant consistent effect was observed on the formation of either uranium acetate-soluble phosphorus,  $\text{CCl}_3\text{COOH}$ -soluble phosphorus, or inorganic phosphorus with  $10^{-3}$  M quinine or quinacrine (Table IV). In several experiments an increase in the  $\text{CCl}_3\text{COOH}$  fraction was observed with  $5 \times 10^{-4}$  M quinine and quinacrine. Since both concentrations are considerably higher than is normally attained in the blood stream, it is obvious that the action of these antimalarials cannot be related to the ribonuclease activity of the parasites.

Since many enzymatic reactions involving phosphate transfer are inhibited by sodium fluoride, the effect of this inhibitor on the breakdown of ribose nucleic acid by hemolyzed normal and parasitized cells was investigated (Table V). 0.01 M NaF stimulated the production of  $\text{CCl}_3\text{COOH}$ -soluble phosphorus as much as 50 per cent (Experiment 2), but had little effect on either uranium acetate-soluble phosphorus or inorganic phosphorus. Manometric studies with bicarbonate buffer and an atmosphere of 95 per cent  $\text{N}_2$ -5 per cent  $\text{CO}_2$  revealed that NaF did not influence the evolution of carbon dioxide when nucleic acid was the substrate, and, therefore, had no effect on the formation of free acid (Fig. 5).

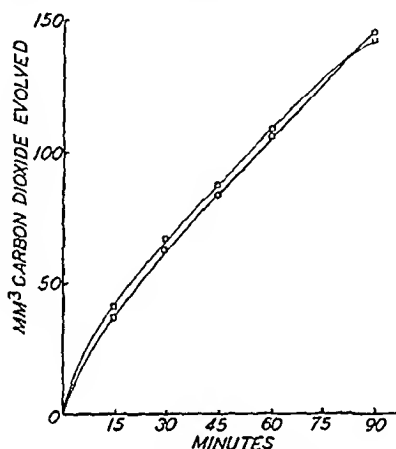


FIG. 5 Effect of NaF on acid production from nucleic acid by hemolyzed, parasitized erythrocytes. O, control, □ 0.01 M NaF.

One experiment (Experiment 8) was performed with a nucleic acid hydrolysate in which all the nucleic acid had been rendered soluble by autoclaving with  $\text{NH}_4\text{OH}$ . Only a slight inhibition by NaF of the production of inorganic phosphate was observed with this preparation as substrate.

0.01 M iodoacetic acid, a typical inhibitor of sulfhydryl enzymes, did not influence the formation of uranium acetate-soluble phosphorus or inorganic phosphorus (Table V).

It has been reported (14) that the inhibitory action of certain diamidines on the growth of bacteria can be prevented by the inclusion of a relatively large amount of nucleic acid in the medium. Moreover, some diamidines have an antimalarial action *in vivo* (15). The effect of stilbamidine on the ribonuclease activity of the hemolyzed red blood cell has therefore been investigated. Since stilbamidine precipitates at the pH usually employed in these experiments, its effect was studied by incubating hemolyzed red cells with solutions of stilbamidine for 30 minutes at a pH of 6.0, washing



thoroughly with saline, and determining the residual activity of the treated preparation towards nucleic acid. Control preparations were carried through precisely the same steps. Inhibition was usually observed with preparations treated with 0.01 M stilbamidine. In some cases, activation was observed after treatment with 0.001 M stilbamidine (Table VI). It is

TABLE V

*Effect of 0.01 M Sodium Fluoride and Iodoacetic Acid on Breakdown of Nucleic Acid by Normal and Parasitized Erythrocytes*

Experiment No	Experiment	Cells per vessel $\times 10^9$	Per cent parasitization	Uranium acetate soluble P	CCl <sub>3</sub> COOH soluble P	Inorganic P
				$\gamma$	$\gamma$	$\gamma$
1	Control	2.0	72	405		21.4
	NaF			415		20.2
	CH <sub>3</sub> ICOOH			500		18.2
2	Control	1.0	86	615	2250	
	NaF			455	3350	
3	Control	2.0	0		1750	
	NaF				1800	
4	Control	2.0	0		2300	
	NaF				2595	
5	Control	3.4	39			49.0
	NaF					36.0
	CH <sub>3</sub> ICOOH					37.0
6*	Control	3.2	87			129
	NaF					90
7*	Control	3.4	77			98
	NaF					107
	CH <sub>3</sub> ICOOH					111
8†	Control	3.4	39			49.0
	NaF					35.9
	CH <sub>3</sub> ICOOH					37.2

Incubated 1 hour at 38°. Buffer, veronal acetate or bicarbonate, pH 7.4. Nucleic acid, 100 mg per vessel.

\* The nucleic acid used in these experiments was not reprecipitated. The rate of formation of inorganic phosphorus was always faster from impure nucleic acid, presumably because of the presence of a greater concentration of intermediate breakdown products.

† The substrate was nucleic acid hydrolysate.

of interest to note that incubation with a very high concentration of stilbamidine, 0.1 M, caused complete solution of the preparation, the resulting product was very viscous and resembled a solution of thymus nucleic acid.<sup>3</sup>

*Effect of Freezing and Thawing*—Hemolysis of the red cell by saponin

<sup>3</sup> This is not too unexpected, since Dounce and Lan (16) have reported that the desoxyribose nucleic acid content of the chicken erythrocyte is 45 per cent.

produces only a slight change in the morphological appearance of the nucleus and the parasite. Most of the nuclei and parasites appear normal when examined after the application of Giemsa stain. It was of interest to determine whether rupture of the nucleus and parasite by freezing and thawing could liberate the enzymes and increase their activity towards ribose nucleic acid. Saponin-hemolyzed preparations were plunged in dry ice-acetone mixtures until frozen, and were thawed slowly at room temperature. This process was repeated three times. The gelatinous mass which resulted was uniformly dispersed in a glass homogenizer. No significant change in activity was noted in either normal or parasitized blood after freezing and thawing.

TABLE VI

*Effect of Preliminary Incubation with Stilbamidine on Ribonuclease Activity of Hemolyzed Red Cells*

Experiment No.	Concentration of stilbamidine	Cells per vessel $\times 10^4$	Percent parasitization	Uranium acetate soluble P		CCl <sub>3</sub> COOH soluble P	
				$\gamma$	percent inhibition*	$\gamma$	percent inhibition
1	0	2.2	78	280		1060	
	$10^{-1}$			372	+33	1420	+34
2	0	2.1	88	330		1120	
	$10^{-1}$			120	64	250	78
	$10^{-2}$			230	30	930	17
	$10^{-3}$					470	58
3	0	2.1	0	90		760	
	$10^{-1}$			40	56	550	28
	$10^{-2}$			60	33	750	1
	$10^{-3}$			100	+10	1000	+32

Incubated 1 hour at 38°, nucleic acid, 100 mg per vessel

\* The plus signs represent stimulation

*Effect of Anaerobiosis*—The degradation of ribose nucleic acid proceeds equally rapidly under aerobic and anaerobic conditions. Measurements of CCl<sub>3</sub>COOH-soluble phosphorus, uranium acetate-soluble phosphorus, and inorganic phosphorus have been made with both normal and parasitized bloods in veronal acetate buffer, pH 7.4, in an atmosphere of oxygen or nitrogen, and in bicarbonate buffer, pH 7.4, in an atmosphere of 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub> or 95 per cent N<sub>2</sub>-5 per cent CO<sub>2</sub>. Manometric studies also reveal no differences.

*Effect of Prolongation of Incubation Period*—Incubation with ribose nucleic acid for long periods of time reveals that the amount of uranium acetate-soluble phosphorus increases steadily over a period of 4 hours (Fig. 6). The curve tends to flatten out somewhat, but the increase is

thoroughly with saline, and determining the residual activity of the treated preparation towards nucleic acid. Control preparations were carried through precisely the same steps. Inhibition was usually observed with preparations treated with 0.01 M stilbamidine. In some cases, activation was observed after treatment with 0.001 M stilbamidine (Table VI). It is

TABLE V

*Effect of 0.01 M Sodium Fluoride and Iodoacetic Acid on Breakdown of Nucleic Acid by Normal and Parasitized Erythrocytes*

Experi- ment No	Experiment	Cells per vessel $\times$ $10^6$	Per cent para- sitization	Uranium acetate soluble P	$\text{CCl}_3\text{COOH}$ soluble P	Inorganic P
				$\gamma$	$\gamma$	$\gamma$
1	Control	2.0	72	405		21.4
	NaF			415		20.2
	CH-ICOOH			500		18.2
2	Control	1.0	86	615	2250	
	NaF			455	3350	
3	Control	2.0	0		1750	
	NaF				1800	
4	Control	2.0	0		2300	
	NaF				2595	
5	Control	3.4	39			49.0
	NaF					36.0
	CH-ICOOH					37.0
6*	Control	3.2	87			129
	NaF					90
7*	Control	3.4	77			98
	NaF					107
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	NaF					35.9
	CH-ICOOH					37.2

Incubated 1 hour at 33°. Buffer, veronal acetate or bicarbonate, pH 7.4. Nucleic acid, 100 mg. per vessel.

\* The nucleic acid used in these experiments was not reprecipitated. The rate of formation of inorganic phosphorus was always faster from impure nucleic acid, presumably because of the presence of a greater concentration of intermediate breakdown products.

† The substrate was nucleic acid hydrolysate.

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*Effect of Freezing and Thawing*—Hemolysis of the red cell by saponin

<sup>3</sup> This is not too unexpected, since Dounce and Lan (16) have reported that the desoxyribose nucleic acid content of the chicken erythrocyte is 45 per cent.

TABLE VII

*Effect of Prolonged Incubation on Formation of  $\text{CCl}_3\text{COOH}$ -Soluble Phosphorus*

Experiment No	Cells per vessel $\times 10^9$	Cells	Per cent parasitization	Time of incubation	$\text{CCl}_3\text{COOH}$ soluble P content	$\Delta\text{CCl}_3\text{COOH}$ -soluble P
				hrs	$\gamma$	$\gamma^*$
1	2 0	Normal	0	1	3150	
				4	1700	-1450
2	2 0	"	0	1	2300	
				4	1600	-700
3	2 5	"	0	1	2760	
				4	2100	-60
4	2 0	"	0	1	1750	
				4	1500	-250
5	1 4	"	0	1	1120	
				4	1340	+220
6	1 4	"	0	1	1260	
				4	1540	+280
7	1 3	Parasitized	48	0 33	1684	
				0 66	2284	
				1	2584	
				2 5	2104	
				4	1204	-1380
8	2 3	"	70	1	2180	
				4	900	-1280
9	1 7	"	73	1	2500	
				4	1600	-900
10	1 8	"	91	1	1650	
				2	1520	
				3	1540	
				4	780	-870
11	1 0	"	86	1	2750	
				4	2000	-750
12	1 3	"	88	1	2000	
				4	1350	-650
13	2 5	"	64	1	3440	
				4	2840	-650
14	1 5	"	55	1	2325	
				4	1725	-600
15	1 4	"	83	1	1880	
				4	1540	-340
16	1 4	"	67	1	2140	
				4	2120	-20
17	1 4	"	65	1	3600	
				4	2640	+40
18	1 6	"	78	1	1850	
				4	1950	+100
19	2 5	"	66	1	2140	
				4	2680	+540

Veronal acetate buffer, pH 7.0, 38°, nucleic acid, 100 mg per vessel

\*  $\Delta\text{CCl}_3\text{COOH}$ -soluble phosphorus represents the difference between the 1 and 4 hour values. A minus sign denotes disappearance of soluble phosphorus, and a plus sign denotes an increase.

steady and consistent Measurement of the  $\text{CCl}_3\text{COOH}$  fraction, however, shows that a peak value is reached within 1 or 2 hours, and that some of the  $\text{CCl}_3\text{COOH}$ -soluble phosphorus which had already formed disappears (Fig 6) In this experiment, a series of vessels, prepared identically, containing 100 mg of nucleic acid, veronal acetate buffer, pH 7.0, and  $2 \times 10^9$  parasitized red blood cells was incubated at  $38^\circ$  At various times vessels were removed, the contents of the vessel were precipitated with uranium acetate solution or  $\text{CCl}_3\text{COOH}$ , and the soluble phosphorus contents of the filtrates estimated

A total of nineteen separate experiments have been performed in which the  $\text{CCl}_3\text{COOH}$ -soluble phosphorus was estimated after 1 and 4 hours (Table VII) Both normal and parasitized cells have been studied In

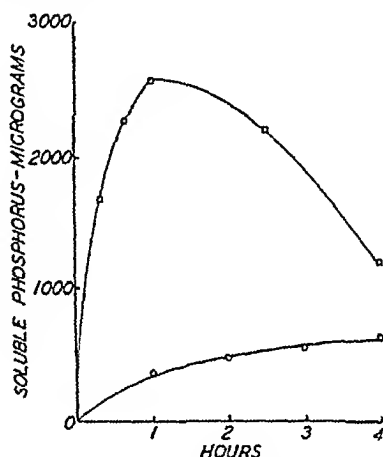


FIG 6 Effects of prolonged incubation on the formation of soluble phosphorus □,  $\text{CCl}_3\text{COOH}$  soluble phosphorus, ○, uranium acetate-soluble phosphorus

fourteen of nineteen experiments, the amount of  $\text{CCl}_3\text{COOH}$ -soluble phosphorus decreased, the decreases ranged from 20 to 1450  $\gamma$  In five experiments the values increased In only one case (Experiment 19) was the increase marked (540  $\gamma$ ) Since blanks on the nucleic acid were conducted in all these experiments, the change cannot be attributed to a reaction occurring independently of the cells

#### DISCUSSION

Our studies reveal that the normal chicken erythrocyte, hemolyzed by saponin, can depolymerize ribose nucleic acid but can effect little further breakdown Since the hemolyzed normal chicken erythrocyte consists essentially of nuclear material, it may be concluded that the cell nucleus catalyzes the depolymerization of ribose nucleic acid This is rather unexpected, since the work of Caspersson and others (17) suggests that the

# ACTIVATION OF SOY BEAN LIPOXIDASE\*

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(Received for publication, May 19, 1947)

Some time ago work in this Laboratory on the oxidation of carotene with lipoxidase and unsaturated fat indicated the presence in soy bean extracts of a heat-stable, protein-like compound which enhanced the activity of the enzyme (1). This observation has been confirmed many times in our laboratory, in fact a purified preparation of "activator"<sup>1</sup> is used routinely in all assays for lipoxidase. The presence of this so called activator in soy beans was demonstrated independently by Theorell, Bergstrom, and Åkeson (2) over a year after our first report of its existence. Subsequently, Cosby and Sumner (3) stated that they were unable to observe any such effect with material from heated soy bean extracts. This discrepancy was found to be due to the inclusion of gum arabic in their substrate, which will be discussed later.

The function of the activating substance, or substances, is admittedly still obscure. It has been observed to protect the enzyme from inactivation by shaking, and also from inactivation during the course of fat oxidation, when a considerable portion of the enzyme was often lost (1). The effect may be mechanical, and it certainly involves the substrate as well as the enzyme, for unless substrate and activator are mixed prior to the introduction of enzyme, no activating effect is noted.<sup>2</sup> With large amounts of activator, inhibition is frequently observed, an effect also noted by Theorell *et al* (2). Partially purified as well as crude preparations of activator produce this effect, but of course inhibitory substances may still be present. For practical purposes, there is thus an optimum dose of activator for a given amount of fat. The ratio of activator to substrate which gives maximum activation in the carotene oxidation procedure is higher than in the oxidation of the primary substrates alone. That activation (or inhibition)

\* Enzyme Research Laboratory Contribution No. 105

<sup>1</sup> The term activator is used here in the sense of a non specific substance which increases the activity of an enzyme, without any preconception as to the mode of action of the substance or substances.

<sup>2</sup> In some cases when the substrate concentration is very low, addition of the activator with the enzyme causes activation, but the effect is never noted if the enzyme is added to the substrate prior to the addition of activator.

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lipoxidase and its activator on the ultraviolet absorption spectrum of linoleic acid, but experiments were made to see whether the activator merely increased the absorption peaks previously noted for enzymatically oxidized linoleic acid by Beigstrom (9), Holman and Burr (10), and Holman (11), or changed the characteristics of the absorption curve. Oxygen uptake, perox-

TABLE III

*Activation of Lipoxidase by Crystalline Material Isolated from Soy Beans*

		Per cent carotene destroyed in 30 sec
Enzyme alone		2
"	+ 0.06 mg crystals	20
"	+ 0.12 " "	26
"	+ 0.30 " "	38
"	+ 0.36 " "	39
"	+ 0.6 " "	40
"	+ 0.1 " regular activator	40

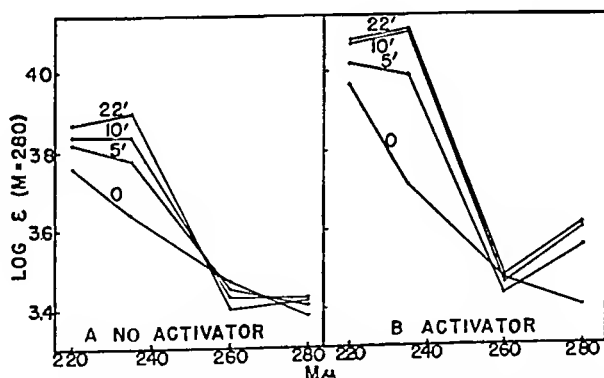


FIG 3 Effect of activator on ultraviolet absorption of enzymatically oxidized linoleic acid. Substrate, 5 mg of linoleic acid, 2.5 ml of 0.05 N NaOH, 45 ml of H<sub>2</sub>O, and 2.5 ml of  $\frac{2}{3}$  M phosphate, pH 6.5. A, 4 ml of substrate, 5.25 ml of H<sub>2</sub>O, and 0.25 ml of enzyme (2 units of lipoxidase); B, 4 ml of substrate, 0.5 ml of activator (0.5 mg), 4.75 ml of H<sub>2</sub>O, and 0.25 ml of enzyme. The instrument used was a Beckman quartz spectrophotometer with a 1 cm cell.

ide formation, and ultraviolet absorption were determined on comparable enzyme substrate mixtures with and without activator. It is shown in Fig 3 that absorption at 235 mμ has merely been increased by the presence of activator, indicating the formation of more of this oxidation product of linoleic acid. The effect of activator on absorption at 280 mμ is more marked than its effect on absorption at 235 mμ. This is seen very clearly if one compares



suggests that the two are not identical, although they both appear to be polypeptide in nature

TABLE II

*Outline of Lipoxidase Purification\* and Isolation of Crystalline Material from Soy Beans Capable of Activating Lipoxidase*

	Units of lipoxidase
I 10% suspension of defatted soy bean meal in 0.2 M acetate buffer, pH 5.0, centrifuged, 2.4 kilos gave 19 liters supernatant	2,720,000
II Supernatant pH 6.5-7.0 and 0.5 M saturated with $(\text{NH}_4)_2\text{SO}_4$ , left in refrigerator overnight, centrifuged, ppt suspended in 3 liters water, centrifuged again and insoluble portion discarded	2,360,000
III Solution dialyzed 60 hrs at 5° with 5 or 6 changes of distilled water (3.75 liters)	1,630,000
IV Adjusted to pH 5.1 with M acetic acid, inert ppt centrifuged out (3.4 liters)	1,700,000 (370†)
70 ml 2% yeast nucleic acid, pH 5.0, added in cold, centrifuged, supernatant neutralized and barium acetate added until supernatant gave no more ppt with excess $\text{Ba}^{++}$ , 15.4 gm $(\text{NH}_4)_2\text{SO}_4$ added and suspension left in refrigerator overnight, centrifuged (3.25 liters)	1,625,000
VI Solid $(\text{NH}_4)_2\text{SO}_4$ added to 0.35 saturation, saturated $(\text{NH}_4)_2\text{SO}_4$ added slowly with mechanical stirring to 0.49 saturation, filtered, ppt dissolved in 76 ml water	950,000
VII Saturated $(\text{NH}_4)_2\text{SO}_4$ added to filtrate to 0.6 saturation, filtered, ppt dissolved in 40 ml water, filtrate discarded	444,000
VIII Solutions from VI and VII dialyzed 48 hrs against cold distilled water	VI, 580,000 (405†) VII, 444,000 (550†)
IX Marked sheen in fraction from VI during dialysis, thin rectangular colorless plates, very insoluble in water, removed from soluble material by centrifuging, more crystals formed when supernatant was left in cold	No lipoxidase activity in crystals
X Supernatant from IX (rod shaped bacteria present)	460,000
Total enzyme recovered from preparation	904,000 (33% of original)

\* The enzyme preparations used in the activation experiments were obtained by methods similar to the one outlined above except that step (V) was omitted. In a typical procedure after ammonium sulfate fractionation the dialyzed protein solution was treated with purothionin or protamine sulfate, which precipitated inert material and usually doubled the specific activity of a given preparation.

† Specific activity as units of lipoxidase per mg of protein nitrogen

*Effect of Activator on Ultraviolet Absorption Spectrum of Enzymatically Oxidized Linoleic Acid*—No attempt was made to study in detail the effect of

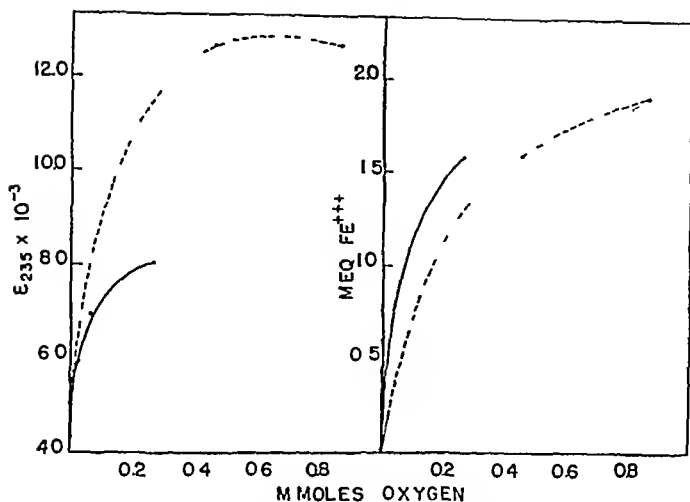


FIG 5 Comparison of oxygen uptake, chromophore production (235  $m\mu$ ), and peroxide formation in lipoxidase-oxidized linoleic acid. The enzyme, activator, and substrate amounts are given in Fig 3 but the solutions used for oxygen uptake and peroxide determinations were not diluted further as indicated there. This dilution was necessary for the spectrophotometric measurements.

TABLE IV  
*Effect of Activator on Ethyl Linoleate Oxidation*

Experiment	Peroxide formation		
	M eq $Fe^{+++}$ per mm ethyl linoleate		
	5 min	15 min	24 min
	12 min	30 min	2½ hrs
A No activator	0.057	0.087	0.096
1 mg activator per 10 mg fat	0.063	0.126	0.144
B No activator	0.428	0.477	0.645
1 mg activator per 2 mg fat	0.300	0.340	0.255
Determined on same solutions used in B	Oxygen uptake		
	mm $O_2$ per mm ethyl linoleate		
	12 min	30 min	2½ hrs
No activator	0.315	0.645	0.780
1 mg activator per 2 mg fat	0.055	0.280	0.405

Experiment A, 1 mg of ethyl linoleate, 0.1 mg of activator, and 0.4 unit of lipoxidase per ml. Experiment B, 0.2 mg of ethyl linoleate, 0.1 mg of activator, and 1.2 units of lipoxidase per ml. The concentrations of buffer and acetone were the same as in Fig 4.

the ratios of  $\Delta\epsilon_{235} : \Delta\epsilon_{280}$  for the activated and unactivated reactions. Without activator the ratios are 8.2, 9.5, and 15.4 at 5, 10, and 22 minutes, respectively, with activator, the corresponding ratios are 4.5, 5.1, and 4.9, respectively. Bergstrom has pointed out that the enzymatic oxidation of linoleic acid differs from autooxidation in the production of some compound which absorbs in this region.<sup>5</sup> Bergstrom's preparations contained activator. When activator was omitted (Fig. 3), the formation of this compound, believed by Bergstrom to be an unsaturated ketone, was negligible.

*Effect of Activator on Oxygen Consumption and Peroxide Formation*—The presence of activator markedly increased the rate of enzymatic oxidation

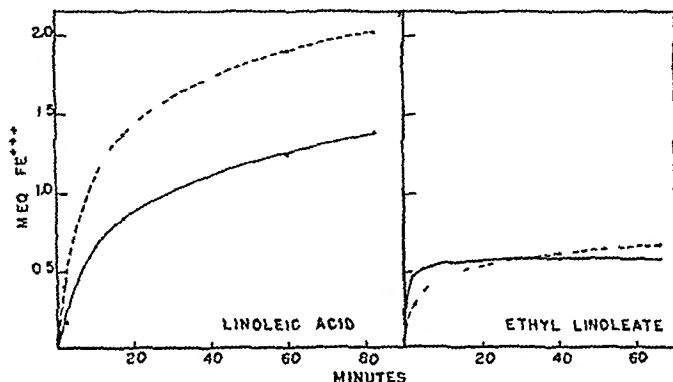


FIG. 4 Effect of activator on peroxidation of linoleic acid and ethyl linoleate with lipoxidase. Peroxide values are expressed as milliequivalents of  $\text{Fe}^{+++}$  per mm of substrate. Substrate, 5 ml of acetone containing 10 mg of linoleic acid or ester, 37.5 ml of  $\text{H}_2\text{O}$ , 2.5 ml of  $\frac{3}{4}$  M phosphate, pH 6.5, and 5 ml of activator solution or  $\text{H}_2\text{O}$  as indicated. Enzyme, 2.5 units of lipoxidase per mg of substrate. Solid lines, without activator, broken lines, with 0.1 mg of activator per mg of substrate. The results were essentially the same if the amount of activator was increased 3 to 5 times, although larger amounts of activator were a little less effective than the amount shown on the graph.

of linoleic acid, as is evidenced by consumption of oxygen (Fig. 1) and the formation of peroxide (Fig. 4). During the course of the enzymatic oxidation of linoleic acid, the ratio of peroxide formed to oxygen absorbed was constant in a given experiment, provided the activator was present in

<sup>5</sup> The formation of a compound absorbing in the region of 270 to 280  $m\mu$  is not necessarily confined to enzymatically oxidized linoleic acid, however. Holman and Burr (12) and Bolland and Koch (13) have observed its formation in autooxidized preparations. Holman (11) states that the only difference he has found between the products of enzymatic oxidation and autooxidation of ethyl linoleate is the increased stability of the former toward alkali. Most authors agree that the compound which absorbs in the region 270 to 280  $m\mu$  is probably a decomposition product of the primary oxidation product.

enzyme when the reaction was followed for several hours. The possible implications of these data will be discussed in a later paper.

Ethyl linoleate forms peroxide much less readily than linoleic acid and in this respect was less responsive to activator. At times a slight amount of activation could be demonstrated, but more often the effect observed was inhibition (Fig. 4 and Table IV).

*Effect of Activator on Carotene Oxidation*—In contrast to the effect of the enzyme on the lipides alone, carotene, when it is present, is oxidized by lipoxidase through ethyl linoleate faster than through linoleic acid (Table V). This may be an example of the observation of Sumner (14) that conditions which slow the rate of peroxidation by the enzyme cause a greater carotene oxidation. In the presence of carotene the activator has been found to accelerate the oxidation in each case, but the oxidation is still not as readily transmitted to carotene through linoleic acid as through its ester.

Peroxide in the ethyl linoleate increases the rate of carotene oxidation by lipoxidase without activator (Table V). The use of highly peroxidized ethyl linoleate as the primary substrate would thus tend to minimize the observed effect of the activator. In the extreme case, no activation at all would be observed. Since the effect of activator on the rate of carotene oxidation with ethyl linoleate is thus similar to the effect of preformed peroxide in the primary substrate, it is believed that the activator has something to do with the formation or the use of fat peroxide.

#### SUMMARY

A crystalline polypeptide which enhances the oxidation of carotene-ethyl linoleate by lipoxidase has been isolated from soy beans, but is apparently different from the amorphous material previously described.

A substance similar to the activator (or activators) present in soy beans has been demonstrated in gum arabic, an exudate from the acacia tree. The use of this gum as an emulsion stabilizer by Cosby and Sumner may account for their failure to demonstrate the activation of purified lipoxidase preparations.

Preliminary experiments suggest that the activator is concerned with the formation of a compound from linoleic acid which absorbs in the region of 280  $m\mu$ .

As shown by the foregoing data, the substance or substances which constitute this "activator" of soy bean lipoxidase may produce under favorable circumstances and with purified enzyme a very great acceleration of the reaction, often amounting to 300 per cent. All of the reactions credited to lipoxidase (oxygen uptake, peroxide formation, and carotene oxidation) appear to be accelerated, but a most puzzling feature is that they are not all accelerated equally, and furthermore there is a marked difference between

optimum amount When activator was omitted, both peroxide formed and oxygen absorbed were less and the increase in peroxide value was no longer proportional to the oxygen absorption Bergstrom also found that the ratio of peroxide value to oxygen uptake was constant in a given experiment (when activator was present and the dose of enzyme was progressively increased) and concluded that all of the absorbed oxygen was present as peroxidic groups We have hesitated to attach much significance to the  $\text{Fe O}_2$  ratios because of the inadequacies of the peroxide method (*cf* Lea (5)) and because the value of the ratio varied from one experiment to another

TABLE V  
*Activation of Carotene Oxidation, Effect of Primary Substrate*

Primary substrate	Activator	Per cent carotene oxidized		
		30 sec	1 min	2 min
A Fresh ethyl linoleate (peroxide value 0.04 m eq per mm)	—	13		
“ “ “ “ “	+	54		
Old ethyl linoleate (peroxide value 1.86 eq per mm)	—	38		
“ “ “ “ “	+	53		
B Fresh ethyl linoleate solution	—	9	16	26
“ “ “ “ “	+	44	78	95
Old ethyl linoleate solution	—	37	50	64
“ “ “ “ “	+	57	82	95
Fresh linoleic acid solution	—	6	7	15
“ “ “ “ “	+	6	10	39
C * Fresh ethyl linoleate solution	—	72	82	90
“ “ “ “ “	+	85	94	(100)
Fresh linoleic acid solution	—	12	37	98
“ “ “ “ “	+	6	20	77

\* 3 times as much enzyme as used in Experiment B

Under the conditions of the experiment described in the previous section, peroxide formation and absorption at  $235 \text{ m}\mu$  were not directly proportional to oxygen uptake, even in the presence of activator (Fig 5) These solutions were extremely dilute, thus allowing more complete oxidation (on the basis of oxygen absorbed per mole of substrate) than is usually observed with lipoxidase This may account for the lack of proportionality Holman (11) has found that absorption at  $235 \text{ m}\mu$  may actually decrease with increased oxygen uptake in enzymatically oxidized fats Recent work in this Laboratory has indicated that a greater degree of peroxide destruction, as well as peroxide formation, occurred with increasing amounts of

obtained in several experiments, although there is considerable variation from one experiment to another in the exact concentrations required for growth. For half maximal turbidity at 24 hours *Lactobacillus arabinosus* required in this experiment the indicated concentration of any one of the following compounds: PABA 0.000035  $\gamma$  per ml, PABG 0.00013  $\gamma$  per ml, pteric acid 0.00026  $\gamma$  per ml, pteroylglutamic acid 0.001  $\gamma$  per ml, pteroyltriglutamic acid 0.023  $\gamma$  per ml, glutamic acid polypeptide of PABA 0.32  $\gamma$  per ml, thymine 13  $\gamma$  per ml. The organism attained maximal growth after 40 hours incubation with 30  $\gamma$  of thymine per ml. Maximal acid production of about 9.5 ml was obtained with each of the compounds

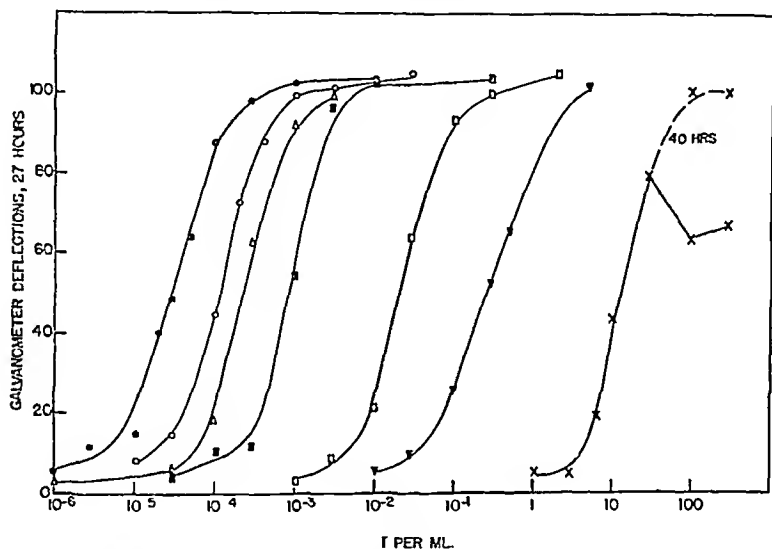


FIG. 1. The response of *Lactobacillus arabinosus* to compounds related to *p*-aminobenzoic acid: ●, *p*-aminobenzoic acid, ○, *p*-aminobenzoylglutamic acid, Δ, pteric acid, ■, pteroylglutamic acid, □, pteroyltriglutamic acid, ▼, glutamic acid polypeptide of *p*-aminobenzoic acid, ×, thymine. Tubes containing no added supplement produced a deflection of 4 to 6.

PABA is therefore the most active compound of the group. The activities of the pteric, pteroylglutamic, and pteroyltriglutamic acids are more difficult to assess because of the occurrence of free arylamine in these preparations (1). In this test the pteric acid sample was 13 per cent as active as PABA, whereas its content of free PABA was 6 per cent. The activity of the pteroylglutamic acid sample (3.5 per cent) is also greater than that predicted from its content of free arylamine (0.5 to 1.0 per cent). Another sample of pteroylglutamic acid which contained less than 0.2 per cent of free arylamine possessed 2 per cent of the activity of PABA. However, it cannot be concluded from these data that these compounds possess

*bacillus arabinosus*, have shown that adenine is a product of the action of an enzyme system in which PABA functions

In view of these reports it was of interest to study the interrelations of sulfonamides and PABA, pteroylglutamic acid, and related compounds in the nutrition of *Lactobacillus arabinosus*. Since certain strains of this organism require preformed PABA for growth (7), the various compounds could be tested for their ability to replace PABA in promoting growth. The antisulfonamide activity of the compounds was also determined. In the later stages of the work it became possible to conduct some preliminary experiments with the strain of *Streptobacterium plantarum* used by Auhagen.

The results obtained with these two organisms are presented in this paper.

#### EXPERIMENTAL

The basal medium, compounds, and general techniques used in this investigation have been previously described (1). Stock cultures of *Lactobacillus arabinosus* 17-5<sup>3</sup> and *Streptobacterium plantarum* 10S<sup>3</sup> were maintained on meat infusion agar. The stock strain of *Lactobacillus arabinosus* was purified by plating several times on the meat infusion agar and selecting colonies which on subculture grew slowly in the absence of added PABA. The strain of *Streptobacterium plantarum* was not purified by plating, since it was desired to duplicate as closely as possible the conditions of Auhagen. Both organisms grew slowly in "AC broth" (Difco) and a significant carryover of PABA was observed when such cultures were used to prepare inocula. In a PABA-free medium the growth of both organisms readily became independent of added PABA. Therefore, the cells to be used as inocula were usually grown in the basal medium (1) containing 0.001  $\gamma$  of PABA per ml. The cells of *Lactobacillus arabinosus* thus obtained attained about 5 per cent of maximal growth after 40 hours in the unsupplemented basal medium, whereas 15 to 20 per cent maximal growth occurred within 24 hours with *Streptobacterium plantarum*. Either organism grew well within 24 hours in the presence of 0.001  $\gamma$  of PABA per ml.

The acid production by the organisms was generally measured in the various experiments. However, such data are included in only a few instances, since the results were in essential agreement with the turbidity data.

#### *Results with Lactobacillus arabinosus*

The growth response of this organism to a series of compounds related to PABA is shown in Fig. 1. The results given are characteristic of those

<sup>3</sup> *Lactobacillus arabinosus* 17-5 is No. 8014 and *Streptobacterium plantarum* 10S is No. 10012 of the American Type Culture Collection.

suggests that PABG is utilized by *Lactobacillus arabinosus* only after conversion to PABA. It is of interest that adaptation to growth in the unsupplemented basal medium appeared to be more frequent in the presence of limiting concentrations of PABG than of PABA.

The effect of the addition of purines on the growth of *Lactobacillus arabinosus* with PABA, pteroylglutamic acid, or thymine is illustrated by the data of Table II. The purines are not essential for growth in the presence of PABA or pteroylglutamic acid. However, other experiments have shown that their addition increases the rate of growth and reduces

TABLE II  
Effect of Purines and Purimidines on Response  
of *Lactobacillus arabinosus* to Thymine\*

Thymine γ per ml	Other supplements		Turbidity readings		
			24 hrs	40 hrs	64 hrs
			0	4	10
	p-Aminobenzoic acid	0.0001	24	105	110
	Pteroylglutamic "	0.01	21	90	106
100			2	5	16
100	Adenine (A)	10	22	80	110
100	Guanine (G)	10	3	35	97
100	Xanthine (X)	10	4	56	103
100	Hypoxanthine (H)	10	32	100	112
100	Uracil (U)	10	2	14	42
100	A, G, X, H, U	5 (Each)	8	61	110
	" " " " "	5 "	3	9	18

\* The purines and uracil were omitted from the basal medium for this experiment.

the quantitative requirement of PABA or pteroylglutamic acid. It has been observed previously that both thymine and a purine are necessary to replace pteroylglutamic acid for the growth of *Streptococcus faecalis* R and *Lactobacillus casei* (1, 9, 10). *Lactobacillus arabinosus*, similarly, did not grow unless both thymine and a purine were present. Hypoxanthine has generally been the most active purine, although growth occurred within 64 hours in the presence of any of the four tested. The addition of uracil permitted some increased growth in this experiment but its action has been irregular in other tests. In view of the variable growth occurring in the control tubes it is uncertain whether or not uracil is able to replace the purines partially.

A detailed study was made of the activity of the compounds related to PABA in antagonizing sulfonamide inhibition of *Lactobacillus arabinosus* SP was chosen for most of these experiments because of its use by Teply



intrinsic growth-promoting activity, since they might be utilized only after degradation to PABA. The growth-promoting activity of the pteroylglutamic acid and of the glutamic acid polypeptide of PABA could well be due to contamination with free arylamine.

While the concentration of thymine required to support growth is relatively high, this activity does not appear to be due to impurities. Two different samples of synthetic thymine have shown identical potency. The acid stability and ether insolubility of the active material also support this conclusion. A solution of thymine (1 mg per ml) in 4 N HCl was autoclaved for 1 hour at 15 pounds pressure. The activity of this solution in supporting the growth of *Lactobacillus casei* or *Streptococcus faecalis* R was not altered by this treatment. Over 95 per cent of the activity of a solution containing 1  $\gamma$  of pteroylglutamic acid per ml was destroyed by

TABLE I

*Growth Activity of p-Aminobenzoic Acid and p-Aminobenzoylglutamic Acid for Lactobacillus arabinosus*

Experiment No	Hrs incubation*	Requirements for half maximal growth moles per liter $\times 10^{10}$		PABG PABA
		PABA	PABG	
I	23 T	4.1	17	4.1
	42 "	1.3	2.7	2.1
	65 "	0.65	1.1	1.7
	65 A	0.65	1.1	1.7
II	19 T	5.2	21	4.0
	27 "	2.3	4.8	2.1
	67 A	0.54	0.68	1.3

\* T, turbidity as growth criterion, A, acid production as growth criterion

this procedure. Also, extraction of a thymine solution (1 mg per ml) five times at pH 3.5 with equal volumes of ether did not change its activity in promoting the growth of the test strain of *Lactobacillus arabinosus* or of a mutant strain of *Escherichia coli* that requires PABA for growth (8). When a solution of 0.1  $\gamma$  of PABA per ml was extracted under these conditions, its activity for either organism decreased more than 90 per cent. Thus the ability of thymine to replace PABA for the growth of *Lactobacillus arabinosus* is probably not due to contamination with PABA or pteroylglutamic acid.

A more detailed comparison of the activities of PABA and PABG is presented in Table I. On a molar basis about 4 times as much PABG was required as PABA at 19 to 23 hours. When growth at 3 days was used as the criterion, the activity of PABG approached that of PABA. This



*et al* (3) in their study of antagonism by liver extracts. Similar results, which will not be presented in detail, have been obtained with SA and SD. Teply *et al* observed a partial antagonism of SP by nicotinic acid and nicotinamide. Under our conditions the inhibition of *Lactobacillus arabinosus* by 10  $\gamma$  of SP per ml was not antagonized by the addition of either compound at concentrations of 0.1 to 300  $\gamma$  per ml.

Table III presents the results obtained with the various antagonists. The concentrations required for maximal growth, i.e. complete antagonism, were about 3 times those indicated for half maximal effect. The action of PABA was competitive over a 1000-fold range of SP concentration. PABG appeared to be at least as active as PABA in antagonizing 1  $\gamma$  of SP per ml. However, when subcultures were made from the washed cells of the various tubes of both series into the unsupplemented basal medium, the results indicated that adaptation had occurred with the lower levels of PABG. That is, good growth occurred in these subcultures within 16 hours. This may be the reason for the apparent high activity of PABG under these conditions. When the concentration of SP was increased the antagonism by PABG became delayed and incomplete. The action of 1000  $\gamma$  of SP per ml was not prevented by the addition of 1000  $\gamma$  of PABG per ml, the highest concentration which was not toxic in the absence of SP. These facts indicate that PABG is utilized by *Lactobacillus arabinosus* only after conversion to PABA and that this conversion is itself prevented by SP concentrations above about 100  $\gamma$  per ml. The data of Table I on the relative growth activity of these compounds are in agreement with this hypothesis.

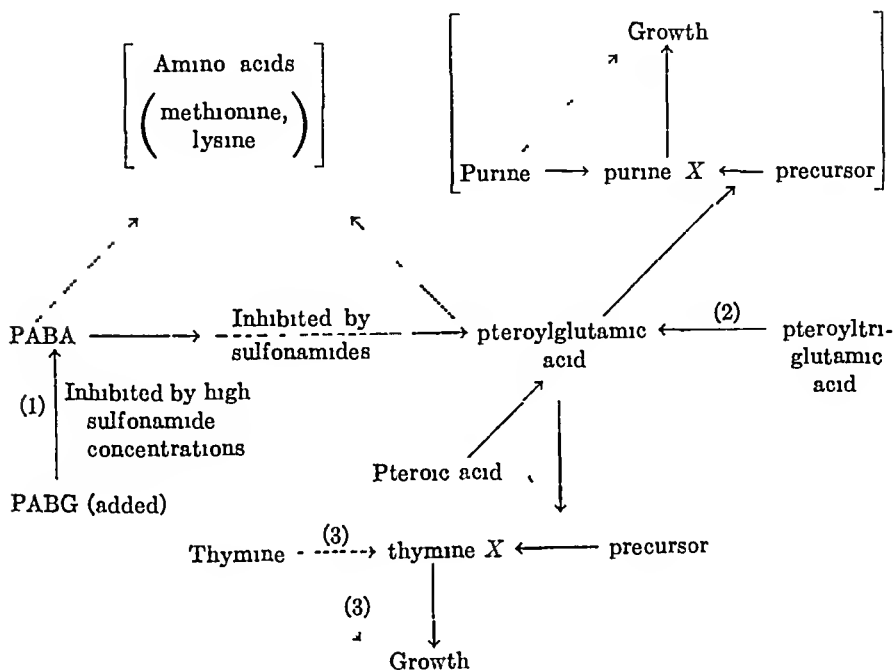
The antagonism by pteroylglutamic acid, pteroylglutamic acid, and thymine is non-competitive. SP is completely inactive in the presence of the indicated levels of these compounds. The pteric acid sample showed competitive antagonism and its activity is approximately that to be expected from its content of free PABA. The slight antisulfonamide activity of the glutamic acid polypeptide of PABA could well be due to traces of free PABA in the sample.

*Lactobacillus arabinosus* was transferred four times in the unsupplemented basal medium to obtain a strain which grew at a normal rate in the absence of PABA, etc., and which presumably is able to synthesize these compounds. The antagonism of the action of SD and SP on such a culture was also studied. The data of the test in which SD was employed are presented in Table IV. The results were similar to those obtained with the parent strain.

#### *Results with Streptobacterium plantarum*

The basal medium and techniques used by Auhagen (4) were duplicated as exactly as possible for the experiments with this organism. The medium

DIAGRAM 1

Possible Relations of *p*-Aminobenzoic Acid and Related Compounds in Bacterial Metabolism

The results obtained thus far with the group of compounds discussed here appear to be explained best by the scheme presented in Diagram 1. Thus pteroylglutamic acid (or a closely related compound) is synthesized from PABA and this synthesis is inhibited by the addition of a sulfonamide. While it is inviting to postulate that either PABG or pterotic acid is an intermediate in this synthesis, the data do not offer support for this hypothesis. In experiments with the enterococci, PABG was inactive in sulfonamide antagonism under conditions in which both PABA and pteroylglutamic acid were active. Also the data of the present paper indicate that added PABG is utilized for growth only after degradation to PABA (reaction (1)). This degradation seems to be of limited occurrence since it is only with the two organisms studied here that PABG has been shown to possess significant activity. The inactivity of pterotic acid in sulfonamide antagonism is also difficult to explain by this hypothesis. With strains of enterococci requiring preformed pteroylglutamic acid for growth, the conversion of pterotic acid to pteroylglutamic acid, which presumably occurs, did not appear to be prevented by sulfonamides. With *Lactobacillus arabi-*

of any one of the following was required PABA 0.00005  $\gamma$  per ml, PABG 0.0005  $\gamma$  per ml, and pteroylglutamic acid about 0.003  $\gamma$  per ml. These results are similar to those obtained with *Lactobacillus arabinosus*.

No explanation can be advanced at present for the discrepancy between our results and those of Auhagen. Some differences in the strain of organism or techniques used probably still exist. However, the relations between PABA, pteroylglutamic acid, and the sulfonamides appear to be the same in *Lactobacillus arabinosus* and *Streptobacterium plantarum*.

#### DISCUSSION

The antagonism of sulfonamide inhibition by PABA is competitive, whereas that observed with pteroyltriglutamic acid, pteroylglutamic acid, and thymine is non-competitive. One must therefore conclude that the organism can use the pteroyl compounds directly in its metabolism. The concentration of thymine required for growth is essentially the same in the presence and in the absence of a sulfonamide. However, the concentrations of the pteroyl compounds required are considerably less in the absence of the sulfonamides. This is probably due, especially with the less active pteroyltriglutamic acid, to the free arylamine which they contain. The growth-promoting activity of this arylamine is competitively prevented by the sulfonamide. Hence the concentration of the pteroyl compounds required for antagonism of the SP is probably the truer measure of their growth-promoting activity. Thus with the organisms studied here, as with the enterococci (1), pteroylglutamic acid, pteroyltriglutamic acid, and thymine appear to be products of enzyme systems in which PABA is involved. Under the conditions employed, the primary point of sulfonamide inhibition must be the synthesis of these substances via PABA.

A puzzling phenomenon is the low activity of pteroylglutamic acid as compared to that of PABA in supporting the growth of *Lactobacillus arabinosus*. A similar situation has been observed with a mutant strain of *Escherichia coli* which requires PABA (8). Here pteroylglutamic acid appears completely inactive in replacing PABA. One possible explanation of the results presented in this paper would be a low permeability of the cells to pteroylglutamic acid. The permeability of many related organisms, which require preformed pteroylglutamic acid for growth, appears adequate, however, this might not be so with the organisms studied here. Another possibility is that the "folic acid" actually produced and utilized by the cell differs in some way from the added pteroylglutamic acid and thus that the added molecule must undergo some conversion before it can be utilized. A third possibility is that PABA might have some function in the cell which can be performed much less efficiently by pteroylglutamic acid. The available data do not permit any decision on this question.

## SUMMARY

The ability of a series of compounds related to *p*-aminobenzoic acid to replace this compound in promoting the growth of *Lactobacillus arabinosus* has been tested. *p*-Aminobenzoylglutamic acid, pteronic acid, pteroylglutamic acid, and pteroyltriglutamic acid are all less active on a molar basis than is *p*-aminobenzoic acid. High concentrations of thymine can replace *p*-aminobenzoic acid under the conditions employed. The addition of a purine is essential for growth with thymine, but is only stimulatory when *p*-aminobenzoic acid or pteroylglutamic acid is present.

The inhibition by sulfonamides of *Lactobacillus arabinosus* is antagonized non-competitively by pteroylglutamic acid, pteroyltriglutamic acid, or thymine. *p*-Aminobenzoylglutamic acid is about as active as *p*-aminobenzoic acid in antagonizing low sulfonamide concentrations, but is much less active against higher concentrations.

*p*-Aminobenzoylglutamic acid and pteroylglutamic acid are less active than *p*-aminobenzoic acid in promoting the growth of *Streptobacterium plantarum*. The inhibition of this organism by sulfonamides is antagonized non-competitively by pteroylglutamic acid or thymine. As with *Lactobacillus arabinosus* the activity of *p*-aminobenzoylglutamic acid approaches that of *p*-aminobenzoic acid against low sulfonamide concentrations but is much less against high concentrations.

The possible relations of the various compounds in bacterial metabolism are discussed. Pteroylglutamic acid, the purines, and thymine are concluded to be products of enzyme systems in which *p*-aminobenzoic acid functions. *p*-Aminobenzoylglutamic acid appears to be utilized by the two organisms only after conversion to *p*-aminobenzoic acid. It is suggested that the product of the action of pteroylglutamic acid is not thymine (nor the purine) as such, but rather some derived or closely related substance.

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*nosus* and those enterococci which are able to synthesize their own supplies of pteroylglutamic acid, this conversion either does not occur or is sulfonamide-sensitive. These results suggest that pteric acid is not a normal intermediate in the synthesis of pteroylglutamic acid but can be converted to it by certain organisms.

It is known that pteroyltriglutamic acid can be hydrolyzed to pteroylglutamic acid (reaction (2)) (13). This hydrolysis appears to be essential before the triglutamic acid or larger conjugates can be utilized by some organisms.

Stokes (14) suggested that folic acid acts as a coenzyme in the synthesis of thymine. Since it has been demonstrated that both a purine and thymine are necessary for the replacement of folic acid in the growth of a number of organisms, the same reasoning should apply to the purine synthesis as well. Hitchings *et al* (15) and Strandkov and Wyss (16) have demonstrated that compounds such as 5-biomouracil or thiothymine can inhibit the growth of *Lactobacillus casei* with thymine as the nutrient, but do not affect the growth when folic acid is present. These workers have suggested that thymine and folic acid may act as alternative nutrients or that thymine may precede folic acid in some synthetic process. The most logical explanation of the various phenomena appears to be that the product of the action of folic acid is not thymine as such but rather some derived or closely related substance (thymine X in Diagram 1). Thus growth with thymine would require a reaction (3) not involved in growth with folic acid. This mechanism would permit growth with thymine to show a different sensitivity to inhibitors than does growth with folic acid. The indicated precursor of thymine X cannot be thymine itself, since growth with thymine does not require folic acid. One cannot state at present whether or not growth with thymine involves the formation of thymine X, therefore, alternative routes for reaction (3) are suggested in Diagram 1. It should be mentioned that the concentrations of thymine, the purines, and the amino acids required for growth would be expected to be much greater than those of substances such as PABA or of pteroylglutamic acid, which act catalytically. In Diagram 1 purine synthesis is suggested to involve similar relations to those outlined for thymine.

PABA has been demonstrated to be involved in the synthesis of certain amino acids, especially methionine (6, 17) and lysine<sup>4</sup>. There are no data available at present to indicate whether or not PABA undergoes a conversion to folic acid before it acts in these syntheses. Therefore, both possibilities are indicated in Diagram 1.

<sup>4</sup>Lampen, J. O., and Jones, M. J., to be published.

# STUDIES WITH BACTERIAL SUCROSE PHOSPHORYLASE

## III ARSENOLYTIC DECOMPOSITION OF SUCROSE AND OF GLUCOSE-1-PHOSPHATE

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(Received for publication, May 23, 1947)

It has recently been shown (1, 2) that the sucrose phosphorylase of *Pseudomonas saccharophila* is essentially a "transglucosidase" capable of transferring "energy-rich glucose" to a variety of acceptors, such as inorganic phosphate, ketose sugars, and L-arabinose. In view of the versatility of the enzyme, it seemed likely that arsenate could be substituted for phosphate as glucose acceptor, especially since arsenate is known to substitute for phosphate in at least one biological reaction (3).

The experiments to be reported indicate that arsenate can act as a substrate for the enzyme since it catalyzes the decomposition of both sucrose and glucose-1-phosphate. Glucose-1-arsenate, which would be expected as a product of these reactions, does not accumulate. Presumably it is immediately hydrolyzed to glucose and arsenate. Since both sucrose and glucose-1-phosphate can serve as glucose donors to the enzyme, the addition of small amounts of arsenate to the phosphorylase causes an "arsenolytic" decomposition of these compounds, which appears as an over-all hydrolytic cleavage.

### EXPERIMENTAL

Phosphate-free sucrose-phosphorylase preparations were obtained from dried cells of *Pseudomonas saccharophila* by repeated reprecipitations with ammonium sulfate from citrate buffer as described previously (1). Such preparations had a negligible phosphatase activity against glucose-1-phosphate and a slight invertase activity. When potassium arsenate was added to the preparations, together with either sucrose or glucose-1-phosphate, a rapid evolution of reducing sugar was observed (see Table I).

In the phosphorytic breakdown of sucrose (Experiment 2), there is a rapid initial production of fructose, which ceases when equilibrium conditions are reached (4). With arsenate instead of phosphate, the production of reducing sugar continues until much more hexose is produced than is equivalent to the amount of arsenate added (Experiments 3, 4). Thus, in Experiment 4, the presence of 10 micromoles of arsenate caused the production of 84 micromoles of reducing sugar in excess of that formed in



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decomposition is due in large part to the inhibitory effect of the glucose released

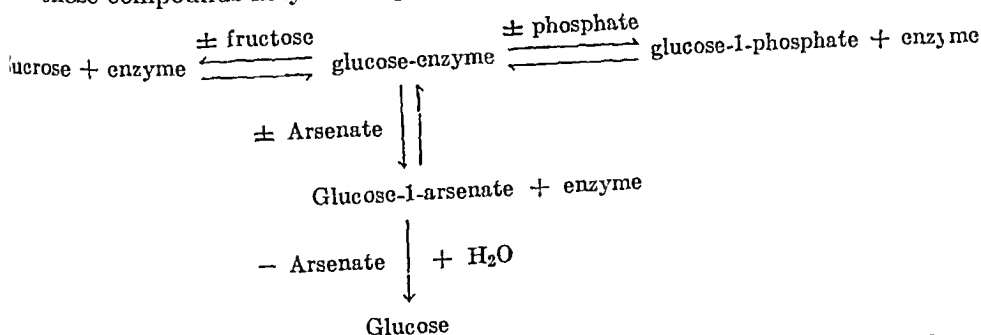
In Experiments 7 and 8, glucose-1-phosphate was substituted for sucrose as glucose donor for the enzyme. It will be seen that after 4 hours twice as much glucose had been produced through the mediation of arsenate as there was arsenate present.

To prove that the reducing values obtained in the decomposition of sucrose and of glucose-1-phosphate were indeed due to the production of hexose sugars, the incubated solutions were passed through ion exchange columns and subjected to fermentation by *Torula monosa*. The reducing sugars were found to be completely fermentable.

#### DISCUSSION

The above experiments show that both sucrose and glucose-1-phosphate can be hydrolyzed by sucrose phosphorylase in the presence of arsenate. Since the intermediate formation of an unstable glucose-1-arsenate can be postulated, it seems appropriate to refer to the breakdown as "arsenolysis". The system enzyme + arsenate becomes a catalyst which behaves both as an invertase and as a phosphatase. Unpublished experiments<sup>1</sup> have shown that the addition of arsenate to potato phosphorylase preparations causes the liberation of glucose from starch.

The demonstration that both sucrose and glucose-1-phosphate can be arsenolyzed, lends further support to the postulated mechanism of action of sucrose phosphorylase. The course of arsenolytic decomposition of these compounds may be simply represented as follows:



The marked decrease in the rate of arsenolysis of both sucrose and glucose-1-phosphate during the course of the reaction can undoubtedly be ascribed to two factors. One of these is the demonstrated inhibition of the reaction by glucose, which is released as an end-product. Glucose appears to compete with the glucose portion of the glucose donors for a

<sup>1</sup> Katz, J., Hassid, W. Z., and Doudoroff, M., unpublished work.

the arsenate-free control (Experiment 1) after 4 hours of incubation. Assuming that 2 molecules of reducing hexose are produced from 1 of sucrose, this represents the decomposition of 42 micromoles of sucrose. The catalytic rôle of the arsenate may also be inferred from a comparison of the data for Experiments 3 and 4, since it can be seen that the rate of sucrose decomposition is almost independent of arsenate concentration in the range tested.

Experiment 6 demonstrates that the sucrose breakdown with arsenate can go to completion (no further increase in reducing value was noted after 4 hours). No disappearance of inorganic arsenate could be detected in these experiments.

TABLE I

*Sucrose Phosphorylase Preparations Reprecipitated from Citrate Buffer*

All experiments except Experiment 6 were carried out simultaneously and with the same preparation. 0.05 M citrate buffer at pH 6.64 was used in all experiments. Glucose-1-phosphate was added as the K salt, adjusted to pH 6.64 with acetic acid. Arsenate and phosphate were added as buffer mixtures at pH 6.64, prepared from  $\text{KH}_2\text{AsO}_4$  and  $\text{KH}_2\text{PO}_4$ , respectively, and NaOH. Incubation at 30°.

Experiment No	Additions, micromoles per ml ( $\pm 2$ per cent)					Reducing sugar produced, micromoles per ml ( $\pm 5$ per cent), after incubation for		
	Sucrose	Glucose 1 phosphate	Glucose	Phosphate	Arsenate	1 hr	2 hrs	4 hrs
1	100					5	11	19
2	100			20		23	27	31
3	100				20	54	79	111
4	100				10	51	74	103
5	100		100		20		32	60
6	25				20			49
7		100				0	1	2
8		100			20	22	31	41

The initial rate of sucrose breakdown with arsenate was found to be almost as great as the rate of phosphorylase. In an experiment of short duration, the former was measured as 92 per cent of the latter. When equivalent amounts of phosphate and arsenate were added together, the initial rate of reducing sugar formation was found to be intermediate between the rates observed with the two substrates separately.

Since it was noted that the rate of reducing sugar formation decreases markedly during the course of the reaction and since it is known that sucrose phosphorylase activity is strongly inhibited by glucose, the effect of added glucose was tested (Experiment 5). On the basis of the results of this experiment, it may be concluded that the decrease in rate of sucrose

# INHIBITORY EFFECT OF KOJIC ACID UPON OXIDATIONS MEDIATED BY LIVER AND KIDNEY\*

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(Received for publication, May 26, 1947)

Administration of increasing doses of kojic acid, 5-hydroxy-2-(hydroxymethyl)-1,4-pyrone, a product of the metabolism of a number of molds and bacteria, produces gastrointestinal disturbances, ataxia, excitement, and convulsions (1). The pattern of electrical activity of brain accompanying the convulsions indicates that they are of the clonic-tonic variety.<sup>1</sup>

In the present work it was found that the rates of oxidation of L-phenylalanine, L-methionine, vanthine, and a number of D-amino acids by rat liver and of L-phenylalanine and D-amino acids by kidney are inhibited *in vitro* by relatively low concentrations of kojic acid.

## EXPERIMENTAL

Liver, kidney, and brain were blended briefly with 1 ml of 0.05 M potassium phosphate, pH 7.8, per gm of tissue and then squeezed through muslin. Muscle was ground in a Latapie mincer with the same proportion of buffer, 8 volumes of buffer were then added to 10 volumes of ground tissue.

The rates of oxygen uptake of 1.8 ml aliquots of the tissue preparations, to which was added 0.2 ml of buffer or buffer containing substrate, inhibitor, or both, were measured at 37° with the usual Warburg apparatus. The pH of the final mixtures was about 7.4. The difference between the oxygen uptake in the presence of substrate and that of its suitable control was considered a measure of the oxidation of the substrate.

The data in Fig. 1 indicate that 0.01 M kojic acid<sup>2</sup> had little effect on the rate of oxygen consumption of skeletal muscle and decreased the rate of oxygen consumption of brain, liver, and kidney considerably. The oxygen uptake of heart, like that of skeletal muscle, was not appreciably affected by the acid. The uptake by liver was inhibited to the greatest extent.

The convulsant dose of kojic acid, when given by intravenous injection,

\* Aided by a grant from the Rockefeller Foundation. A preliminary report of this work has appeared (*Federation Proc.*, 6, 267 (1947)).

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<sup>1</sup> Unpublished observations made in this laboratory.

<sup>2</sup> Kindly supplied by Dr. Frank H. Stodola.

position on the enzyme. The other factor contributing to the decrease in rate must be the competition of fructose and of inorganic phosphate which are produced in the respective reactions with arsenate for combination with the glucose-enzyme complex.

#### SUMMARY

The addition of arsenate to bacterial sucrose phosphorylase catalyzes the hydrolytic decomposition of sucrose and glucose-1-phosphate, presumably through the formation of an unstable compound, glucose-1-arsenate.

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and xanthine by liver. Compared on the basis of concentration of acid producing an inhibition of 0.5, the oxidation of methionine was most sensitive to the presence of the acid and that of tyrosine the least. The rates of oxidation of L-phenylalanine, D-phenylalanine, and DL-alanine by kidney were inhibited by kojic acid to about the same extent as with liver. The rates of oxidation by kidney and liver of DL-leucine and DL-isoleucine, the L forms of which, like L-alanine, were not oxidized by the kidney and liver preparations, were inhibited by the acid to about the same degree as the rate of oxidation of DL-alanine and D-phenylalanine.

Two of the enzymes inhibited by kojic acid, namely the xanthine and D-amino acid oxidases, are flavoproteins. The L-amino acid oxidase of kidney and liver is also a flavoprotein (3). Whether the oxidations of the L

TABLE I

*Inhibitory Effect of Kojic Acid upon Oxidations Effected by Liver*

The rates of oxidation, expressed in micromoles of oxygen per 100 minutes, are the differences in oxygen uptake of the liver preparation in the presence and absence of the substrates. The concentration of kojic acid giving an inhibition of 0.5 was determined by extrapolation from concentrations producing inhibitions less and greater than 0.5. The concentration of DL-alanine was 0.06 M, the concentration of the other substrates was 0.03 M.

Substrate	Rate of oxidation	Concentration of kojic acid producing inhibition of 0.5
	micromoles O <sub>2</sub> per 100 min	M × 10 <sup>-4</sup>
L-Methionine	5.0	4
L-Phenylalanine	7.6	12
D-Phenylalanine	11.2	12
DL-Alanine	10.1	16
Xanthine	10.9	70
L-Tyrosine	6.0	800

forms of methionine, tyrosine, and phenylalanine by the liver and kidney preparations used in the present work are mediated by the L-amino acid oxidase is not clear. For oxidation of L-leucine, estimated from oxygen uptake and ammonia formation, by the liver and kidney preparations was negligible, whereas L-leucine is of all L-amino acids most rapidly oxidized by the purified L-amino acid oxidase (3). However, it is possible that the inhibitory effect of kojic acid on the oxidation of the L forms of methionine, phenylalanine, and tyrosine found in the present work also represents inhibition of a flavoprotein.

The degree of inhibition produced by the acid in the case of each substrate considered above did not depend upon the sequence of addition of substrate and inhibitor to the tissue. Also, the degree of inhibition was not a linear

is about 0.3 gm per kilo of body weight, which corresponds to a blood and total tissue concentration of about 0.05 and 0.004 M respectively. Thus, the possible range of concentration attained *in vivo* with the convulsant dose is of the order of concentration, e.g. 0.001 M, which appreciably inhibits the oxygen uptake of brain *in vitro*. A somewhat similar relation between the convulsant dose of picrotoxin and the concentrations which *in vitro* inhibit the oxygen uptake of brain has been pointed out previously (2)

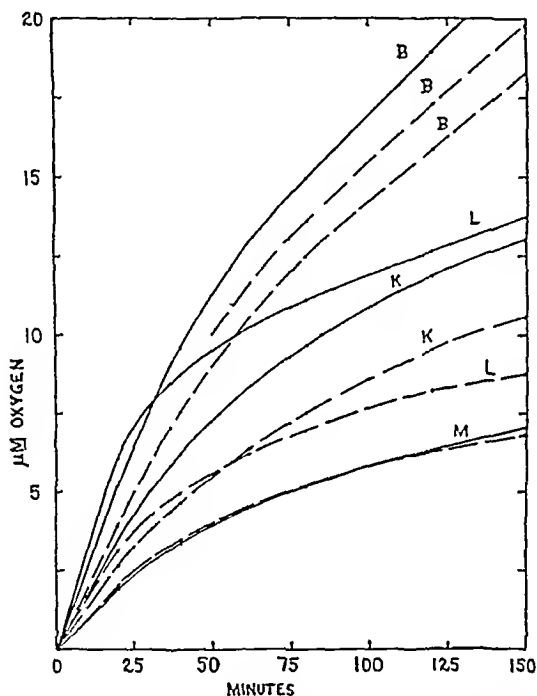


FIG. 1 Effect of kojic acid upon oxygen uptake of brain (B), kidney (K), liver (L), and muscle (M). The solid and broken curves represent respectively the oxygen uptakes of the tissues in the absence and presence of kojic acid, 0.01 and 0.001 M in the case of brain, 0.01 M in the case of the other tissues.

Whether a depression of cerebral oxygen uptake plays a rôle in seizures produced by convulsant drugs is of course not certain. Since kojic acid has no considerable effect on the oxygen consumption of heart muscle *in vitro*, it is of interest that the electrical activity of heart is little affected by the convulsant dose.<sup>1</sup>

The data in Table I indicate that kojic acid inhibits the rate of oxidation of L-methionine, L-phenylalanine, L-tyrosine, D-phenylalanine,<sup>2</sup> DL-alanine,

<sup>1</sup> Kindly supplied by Dr. Clarence P. Berg.

legend of Fig 2) produced by 0.0001 M kojic acid, benzoate, quinine, and atabrine were 0.53, 0.32, 0.04, and 0.05 respectively. Kojic acid is therefore a more effective inhibitor of the oxidase than the other substances.

It has been stated (6) that competitive inhibition of the D-amino acid oxidase is exhibited to some extent by compounds containing system (I). However, fumarate does not inhibit the oxidase in reasonable concentration,

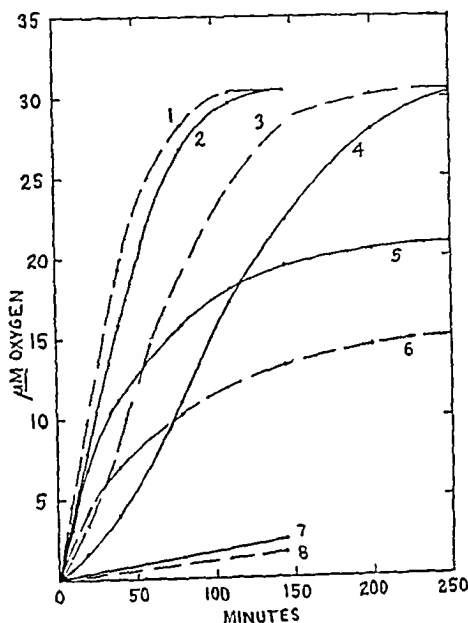


FIG 3 Effect of kojic acid on the oxidation of succinate by liver. The solid and broken curves represent data obtained respectively in the absence and presence of kojic acid, 0.005 M. The oxidation of succinate, 0.03 M, 2 ml total volume, is expressed as the difference between oxygen uptake in the presence of succinate and the uptake of a suitable control. Curves 1 and 2 represent the oxidation of succinate by the washed liver preparation (cf the text), Curves 7 and 8 the oxygen consumption of the controls. Curves 3 and 4 represent the oxidation of succinate by the unwashed liver preparation, Curves 5 and 6 the uptakes of the controls.

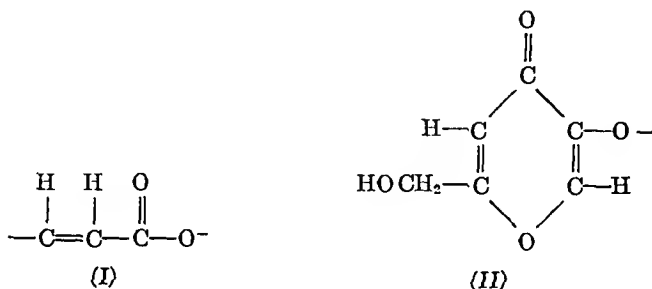
eg 0.005 M, and the ion of kojic acid (II) is not obviously related to (I). Thus, to attribute special significance to a relation between (I) and the presumed characteristic configuration of the enzyme which permits activity and reversible inhibition seems without more than casual merit.

The rates of oxidation of the following substances by the tissues indicated were not inhibited by 0.005 M kojic acid: succinate by muscle, liver, kidney, and brain, tyramine and isoamylamine by brain, kidney, and liver, L-proline, hydroxy-L-proline, choline, and uric acid by liver.

In the case of the substances whose oxidation by brain, liver and kidney



was not inhibited by kojic acid, increases in rate of oxidation were consistently produced by the presence of kojic acid. The oxidation of succinate by muscle was not so affected. The oxidation of succinate by liver, depicted in Fig 3, illustrates this effect. It is possible that the increase in rate is attributable to depression by kojic acid of the activity of systems competing in some way with the oxidative systems in question. This possibility was tested in the case of succinate.



The usual preparations of liver, kidney, and brain were centrifuged, and the precipitates washed in the centrifuge with 0.05 M potassium phosphate, pH 7.4, and finally suspended in enough buffer to restore the original volumes. The effect of this procedure on the oxidation of succinate by liver, which is representative of the effects on the other tissues, is indicated in Fig 3. The pertinent points are that kojic acid still inhibited the oxygen uptake of the washed control, and the increase in rate of oxidation of succinate produced by kojic acid, while decreased by washing, still persisted. It seems, therefore, that the increase in rate of oxidation of succinate produced by the acid depends upon depression of uptake of the control. These points, coupled with the fact that the oxygen uptake of muscle and its oxidation of succinate were not appreciably affected by kojic acid, suggest that in the case of succinate, and presumably the other substrates, the increase in rate of oxidation effected by the acid is consequent upon depression of competing systems.

#### SUMMARY

Kojic acid in relatively low concentrations, *in vitro*, inhibits the oxidation of D-amino acids, xanthine, L-phenylalanine, and L-methionine by liver and the oxidation of D-amino acids and L-phenylalanine by kidney. Oxidation of L-tyrosine by liver is inhibited by relatively high concentrations of the acid. The acid competes reversibly with the substrate for the D-amino acid oxidase. The inhibition of the other oxidations also appear to be of the competitive type.

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# A CRITICAL STUDY OF PROPOSED MODIFICATIONS OF THE ROE AND KUETHER METHOD FOR THE DETERMINATION OF ASCORBIC ACID, WITH FURTHER CONTRIBUTIONS TO THE CHEMISTRY OF THIS PROCEDURE\*

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(Received for publication, June 17, 1947)

In a report (1) to this *Journal*, Bolomey and Kemmerer proposed the substitution of glacial acetic acid for the 85 per cent sulfuric acid used to develop color in the Roe and Kuether method (2) for the determination of ascorbic acid. In a second paper (3) Bolomey and Kemmerer published spectrophotometric studies of the Roe and Kuether method in which they omitted the use of an indispensable reagent, thiourea, and did not recognize that the discrepancies in their absorption curves were due to a departure from the chemical principles published for this method. In this paper we shall present data showing that the modifications proposed by Bolomey and Kemmerer introduce errors that greatly diminish the specificity of the original procedure.

## EXPERIMENTAL

The 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid was prepared by the technique of Roe and Kuether from solutions of pure ascorbic acid and orange juice oxidized with nitrite and with bromine. Derivatives obtained by the same procedure were also made from D-glucose, D-fructose, D-arabinose, and reductones. The reductones were made by boiling a 1 per cent solution of D-glucose in 0.5 per cent  $\text{NaHCO}_3$  for 30 minutes. The derivatives were removed from the solution of reactants by centrifugation and washed twice with distilled water. The washed product was dissolved in 50 per cent sulfuric acid or 50 per cent acetic acid, and the resulting colored solutions were read in a Coleman spectrophotometer, model 10-S. 50 per cent concentration of acid was used, as this is the final concentration of the colored solution in the Roe and Kuether method or the Bolomey and Kemmerer modification. Identical amounts of the derivatives were used when comparison was made of the absorption in acetic and in sulfuric acid.

\* Aided by a grant from the Nutrition Foundation, Inc.

## DISCUSSION

*Sulfuric Versus Acetic Acid*—In Fig 1, *A* and *B*, are shown the spectrophotometric absorption curves of the bis-2,4-dinitrophenylhydrazone of dehydroascorbic acid obtained by oxidation of pure ascorbic acid solution and orange juice with norit and with bromine, coupling being carried out in the presence of thiourea. The curves closely parallel each other, showing

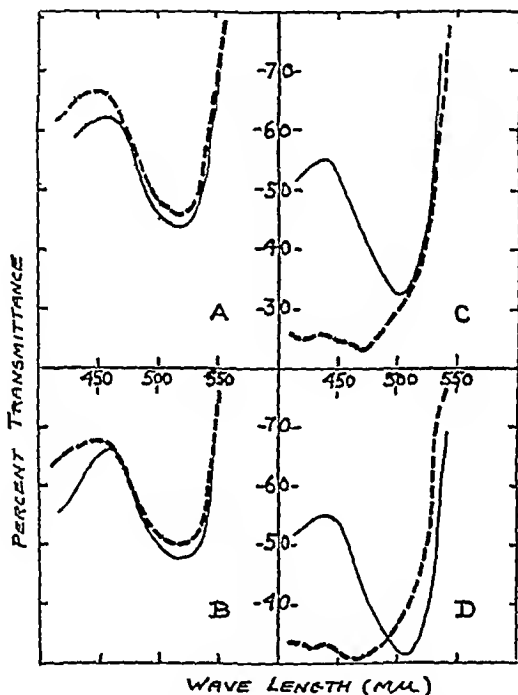


FIG 1 Absorption curves of 2,4-dinitrophenylhydrazine derivatives, prepared as follows *A*, norit oxidized, thiourea present, color developed with 50 per cent sulfuric acid, *B*, bromine oxidized, thiourea present, color developed with 50 per cent sulfuric acid, *C*, norit oxidized, thiourea present, color developed with 50 per cent acetic acid, *D*, bromine oxidized, thiourea present, color developed with 50 per cent acetic acid. The solid line represents the dehydroascorbic acid derivative, the dotted line, the orange juice derivative

that the colored product obtained by treatment of the 2,4-dinitrophenylhydrazine derivatives with sulfuric acid is the same, regardless of whether these derivatives are prepared from pure ascorbic acid solution or from orange juice, by norit or by bromine oxidation. In Fig 1, *C* and *D*, the curves obtained by substituting acetic acid for sulfuric acid are markedly dissimilar. These curves show that substances other than ascorbic acid are present in orange juice which yield derivatives with 2,4-dinitro-

phenylhydrazine that absorb markedly between wave-lengths of 400 to 500  $m\mu$  when acetic acid is used as the solvent. The fairly close agreement of the curves in *C* and *D* at 520  $m\mu$  is fortuitous and does not adequately support the suggestion of Bolomey and Kemmeier that acetic acid may be substituted for sulfuric acid in this method if a 520  $m\mu$  filter is used.

In Fig 2 are shown the spectrophotometric absorption curves of the 2,4-dinitrophenylhydrazine derivatives of D-glucose, D-fructose, D-arab-

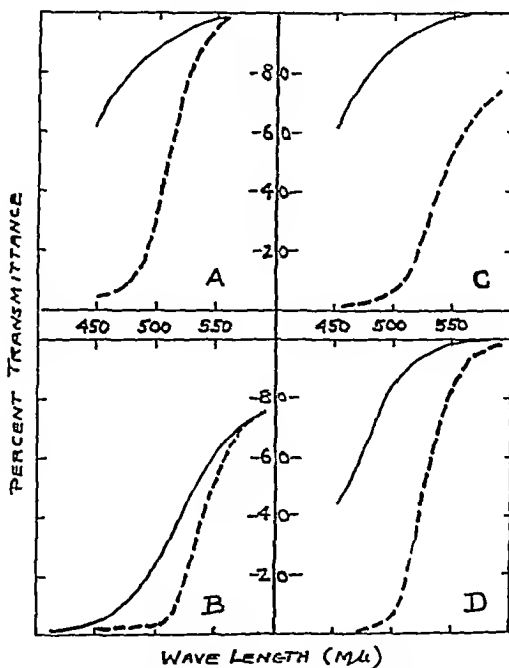


FIG 2 Absorption curves of 2,4 dinitrophenylhydrazine derivatives, prepared as follows: *A*, fructose, 1 per cent solution, coupled 24 hours at 37°, *B*, reductones, 1 per cent glucose, 0.5 per cent  $\text{NaHCO}_3$ , boiled 30 minutes, coupled 3 hours at 37°, *C*, glucose, 1 per cent solution, coupled 24 hours at 37°, *D*, arabinose, 1 per cent solution, coupled 24 hours at 37°. The solid line represents sulfuric acid, the dotted line acetic acid.

inose, and reductones when treated with 50 per cent sulfuric acid and with 50 per cent acetic acid. For each of these derivatives the absorption is much greater in acetic acid solution than in sulfuric acid solution. At the 520  $m\mu$  wave-length the contrast is very marked and shows the possibility of great error from using acetic acid as a solvent in this method.

The superiority of sulfuric acid as a reagent in this method rests upon the fact that it is a reactant as well as a solvent. When sulfuric acid of the proper concentration is mixed with 2,4-dinitrophenylhydrazine de-

derivative of dehydroascorbic acid, a chemical reaction takes place, forming a reddish colored product. This reaction is fairly specific for derivatives of compounds closely similar in structure to dehydroascorbic acid. Most interfering substances that couple with 2,4-dinitrophenylhydrazine form derivatives that do not give a reddish colored product with sulfuric acid. When derivatives of glucose, fructose, and arabinose, for example, are treated with sulfuric acid, they at first dissolve, giving some brownish color to the solution, but this color fades until little or no interference occurs at the 540  $m\mu$  wave-length. This fading is due to the action of the sulfuric acid which apparently splits the compound at the hydrazine linkage with the formation of the original uncoupled products. This is the reason that it was recommended that the color should not be read until at least 30 minutes after its production. On the other hand, when acetic acid is used as a solvent, the derivatives of many, if not all, interfering compounds do not undergo decomposition, as shown by a constancy of the colorimetric reading of their products. This difference in chemical behavior explains the widely diverging absorption curves of the derivatives prepared from orange juice when treated with sulfuric acid and with acetic acid.

*Importance of Thiourea*—In their original paper (2) Roe and Kuether stressed the necessity of thiourea as a reagent in their method. Oxidants such as  $Fe^{+++}$  ions, or  $H_2O_2$ , will produce a color with 2,4-dinitrophenylhydrazine. This method is an empirical procedure in which the coupling of dehydroascorbic acid with 2,4-dinitrophenylhydrazine does not go to completion. The rate of coupling is dependent upon the oxidation-reduction state of the solution and to control it completely, as well as to prevent the effect of oxidants, it is necessary to carry out the coupling reaction in the presence of a known excess of some reducing reagent. If thiourea is not used, another reducing agent, such as  $SnCl_2$ , may be substituted, provided a new calibration curve is made for the revised conditions.

The importance of thiourea is demonstrated by the curves of Fig 3 in which this reagent was not used. In Fig 3, A, the curves for the derivatives prepared from pure ascorbic acid and orange juice parallel each other fairly well when norit was used as the oxidant and  $H_2SO_4$  was added to develop the color, but in B, where bromine was used as the oxidizing agent, the curves are widely separated. This discrepancy was even more marked in the experiments shown by C and D in which acetic acid was used as a solvent of the derivatives. Lowry, Lopez, and Bessey (4) observed slightly higher results in the vitamin C content of blood serum when they used bromine as oxidant instead of norit. The more specific result with norit is apparently due to the adsorption of interfering substances by this reagent. In our work high values from bromine oxidation of orange juice

were prevented, however, by the use of thiourea, as shown in Fig 1, *A* and *B*

In view of the fact that Bolomey and Kemmerer used acetic acid instead of sulfuric acid and omitted the use of an essential reagent, thiourea, their

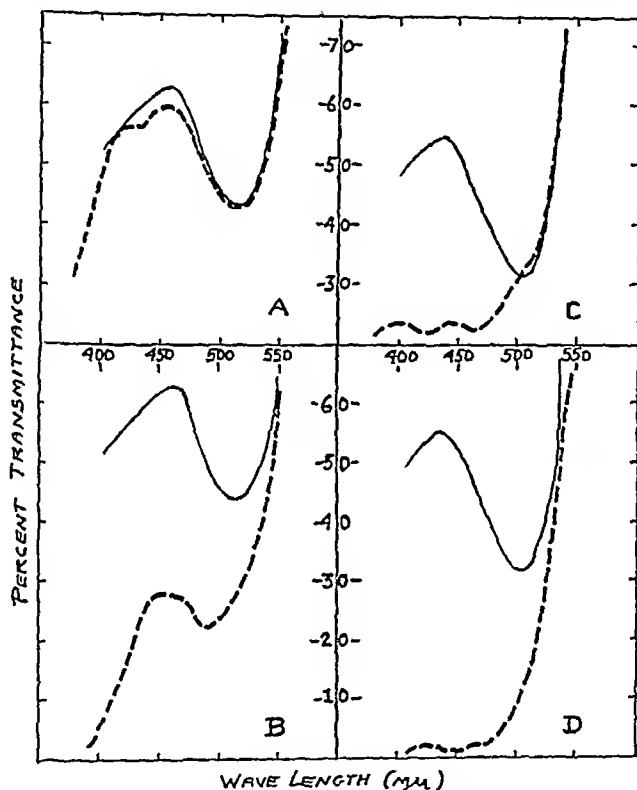


FIG 3 Absorption curves of 2,4 dinitrophenylhydrazine derivatives, prepared as follows *A*, norit oxidized, no thiourea present, color developed with 50 per cent sulfuric acid, *B*, bromine oxidized, no thiourea present, color developed with 50 per cent sulfuric acid, *C*, norit oxidized, no thiourea present, color developed with 50 per cent acetic acid, *D*, bromine oxidized, no thiourea present, color developed with 50 percent acetic acid. The solid line represents the dehydroascorbic acid derivative, the dotted line, the orange juice derivative

published absorption curves (3) represent values given by a composite group of 2,4-dinitrophenylhydrazine derivatives of dehydroascorbic acid and interfering substances, and hence have little significance. Their suggestion that a corrected curve be made by subtracting unoxidized from oxidized filtrate values is pointless, since such differences are not



observed when the technique of Roe and Kuether, as originally published, is followed

*540 or 520 m $\mu$  Filter*—It is clear from the above data and discussion that the effect of interfering substances is very largely prevented by the chemical technique developed. However, selection of the right filter will contribute to the specificity of this method. The absorption maxima of derivatives of most interfering substances are in the region of the shorter wave-lengths. For this reason Roe and Kuether adopted the use of the 540 m $\mu$  filter. The absorption curve of the dehydroascorbic acid derivative has considerable slope in this region but perfect linearity in calibration curves is obtained with the amounts of dehydroascorbic acid used in this method. It is our opinion that the 540 m $\mu$  filter is preferable for analysis of plant and animal tissues in general. In special work, in which it is known that little or no interfering substance is present, the 520 m $\mu$  filter may be used to advantage. The 520 m $\mu$  filter is more satisfactory, for example, in the micromethod for ascorbic acid in blood, developed by Lowry, Lopez, and Bessey (4), because greater absorption occurs at the 520 m $\mu$  wave-length and blood does not contain interfering substances in amounts that will produce error.

#### SUMMARY

Data have been presented showing the possibility of great error from the Bolomey and Kemmerer modifications of the Roe and Kuether method for the determination of ascorbic acid. Further contributions to the chemistry of this method are made.

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# A METHOD FOR THE DETERMINATION OF MANNITOL IN PLASMA AND URINE\*

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(Received for publication, June 20, 1947)

Concurrent analyses of mannitol in urine solutions and protein-free plasma filtrate are used in the measurement of renal function

Two types of procedures have been used in determining mannitol concentration. One depends on the reduction of ferricyanide by mannitol in strongly alkaline solution and requires a correction for the glucose content of the sample (2). The other (3) disposes of glucose by treatment with yeast. In it the total periodate consumed after oxidation in hot acid solution is determined by titration. This method is more specific, as periodic acid oxidizes only compounds with 2 adjacent partially oxidized carbon atoms. It has the further advantage that it does not require a separate determination of glucose. Correction for non-mannitol reducing substance is made from a mannitol-free blank sample. However, the high blank obtained from materials treated with yeast and the fact that the oxidation consumes only 1 of the 4 titratable oxygen atoms of periodate impair its sensitivity.

There are many substances oxidizable by periodate (4). Among them, those with 2 adjacent partially oxidized carbon atoms at the end of the chain yield formaldehyde. Thus, oxidation of 1 mole of mannitol with periodic acid yields 2 moles of formaldehyde and 4 of formic acid. MacFadyen (5) has recently developed a sensitive procedure for determination of formaldehyde. It therefore seemed feasible to develop a more sensitive and specific method for the determination of mannitol in biological media, in which the determination is based on measurement of formaldehyde produced during periodic acid oxidation.

In attempting to apply this principle, there are serious drawbacks to treating the plasma filtrates and urine dilutions with yeast. With plasma this treatment increased the blank rather than decreased it, and the blank of glucose solutions so treated is also materially increased (6). Moreover, in our hands, mannitol was sometimes lost, apparently by absorption on the yeast. We therefore altered the conditions of the oxidation in order to make it more specific and thus to diminish the plasma and glucose blanks.

Mannitol is oxidized by periodic acid at room temperature in neutral or

\* A preliminary report has been published (1)

acid solution, while the oxidation of many other substances, including glucose, proceeds rapidly only under other conditions. We therefore selected a concentration of periodic acid sufficient to oxidize mannitol in the concentrations best suited for color measurement, allowing for the presence of oxidizable, non-chromogenic material, chiefly glucose.

### *Method*

*Principle*—Mannitol is oxidized by periodic acid to formic acid and formaldehyde. The conditions of the oxidation are such that glucose, although attacked, produces little formaldehyde. The periodic acid is reduced to iodide by stannous chloride and the formaldehyde produced is determined by the method of MacFadyen.

#### *Reagents*—

1 Periodic acid reagent. Potassium periodate, 0.03 M in 0.25 M sulfuric acid.

2 Stannous chloride. Prepared freshly every day as approximately 0.125 M in 0.3 N HCl. This solution should be titrated by the periodate reagent immediately before use and so adjusted that 10 cc. of  $\text{SnCl}_2$  reagent titrate 10.2 cc. of  $\text{HIO}_4$  reagent. For the titration, 5 cc. of concentrated HCl are added to the  $\text{SnCl}_2$  and starch is used as the indicator.

3 Chromotropic acid reagent. 0.2 gm. of chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid) is dissolved in 4 cc. of water in a 100 cc. volumetric flask and made up to volume with 15 M sulfuric acid.

*Procedure*—Pipette into a tall test-tube graduated at 25 cc., 2 cc. of plasma filtrate or urine dilution. Plasma filtrate is prepared as a 1:20 dilution of plasma by the method of Somogyi (7). Filtrates made with zinc sulfate and sodium hydroxide or cadmium sulfate and sodium hydroxide are equally satisfactory. Urine dilutions are made with water. In both cases the 2 cc. should contain 0.5 to 3.0 mg. of mannitol per 100 cc. and not more than 7 mg. of glucose per 100 cc. A reagent blank is made containing 2 cc. of distilled water. From a filtrate of mannitol-free plasma two tubes are prepared. One (oxidized blank) is carried through the procedure as described below. The other (unoxidized blank) is treated with stannous chloride prior to addition of periodic acid.

0.5 cc. of periodic acid reagent is added to the oxidized blank and sample tubes and the contents mixed and allowed to stand at room temperature for 8 to 10 minutes.

Add 0.5 cc. of stannous chloride and shake well. The stannous chloride is oxidized by the periodate to stannic acid, which appears as a milky precipitate in the tube. This precipitate is soluble in the strong acid to be added in the next step and does not interfere with the determination. There may also be a momentary appearance of elementary iodine in the

simultaneous plasma mannitol, inulin, creatinine, and thiosulfate clearances is shown in Table II

### DISCUSSION

Any substance oxidized by periodic acid may interfere with the determination. The interference may arise either from the formation of formaldehyde from substances other than mannitol or from excessive consumption of periodic acid. Among the compounds that may interfere because of formaldehyde production are fructose,  $\alpha$ -glycerophosphate, ethylene and propylene glycol, and most compounds which contain either a  $\text{—CHOH—CH}_2\text{OH—}$  or a  $\text{—CO—CH}_2\text{OH—}$  grouping. Substances containing a  $\text{—CHOH—CHOH—}$  or a  $\text{—CO—CHOH—}$  or a  $\text{—CO—CO—}$  group are oxidized but do not yield formaldehyde. These may only interfere with the determination if they are present in such concentrations as will decrease the periodic acid content so that excess stannous chloride begins to reduce formaldehyde.

Of the interfering substances present in blood and urine the most important is glucose. In acid solution, glucose exists chiefly as the pyranose ring form which does not have a free  $\text{—CHOH—}$  group adjacent to the terminal  $\text{—CH}_2\text{OH}$  group. Therefore, under the above conditions the terminal link in the carbon chain is very little oxidized in 10 minutes (formaldehyde production about 5 per cent of theoretical). It is possible therefore to determine mannitol accurately in the presence of small and constant concentrations of glucose by the above procedure. Thus, at concentrations of glucose of about 10 mg per cent in plasma filtrate, recovery of added mannitol averages 95 per cent and at glucose concentrations of 20 mg per cent it averages about 90 per cent. Several factors participate in this, of which the most obvious are (a) inequality of formaldehyde liberation from glucose as between mannitol-containing and mannitol-free samples, which is possibly due to (b) inequalities of periodic acid consumption and stannous chloride excess as between such samples. The difficulty may be largely obviated by addition of mannitol to the mannitol-free, glucose-rich plasma or urine samples and determination of unknowns from the curve thus established.

*Plasma Blank*—The normal plasma blank is equivalent to 3 to 5 mg of mannitol per 100 cc. About half of this blank is due to glucose. It therefore varies during wide fluctuations of the plasma glucose, but in the absence of these is constant in any person over periods of several hours.

*Urine Blank*—The average blank found in undiluted urine is about 0.3 to 0.5 mg per minute. We think it likely that the bulk of this urinary blank is due to the non-fermentable reducing substance normally found in urine.

*Time of Oxidation*—Under the conditions employed, oxidation of man-

TABLE I  
*Recovery of Mannitol Added to Plasma*

Mannitol added	Apparent mannitol	Mannitol recovered	Recovery
<i>mg per 100 cc</i>	<i>mg per 100 cc</i>	<i>mg per 100 cc</i>	<i>per cent</i>
0	3 8		
20	23 6	19 8	99
40	43 6	39 8	99 5
60	63 8	60 0	100
0	4 0		
20	23 6	19 6	98
60	63 4	59 4	99
0	2 4		
20	22 0	19 6	98
40	42 4	40 0	100
60	62 4	60 0	100
0	3 8		
20	23 6	19 8	99
40	43 6	39 8	99 5
60	63 2	59 4	99

TABLE II  
*Ratios of Simultaneous Plasma Clearances of Mannitol, Inulin, Creatinine, and Thiosulfate in Dogs and Human Beings*

The simultaneous plasma clearances of substances believed to be excreted by glomerular filtration are compared in observations made in dogs and human beings, during intravenous infusion of the substances studied. Inulin was determined by the method of Corcoran and Page (8), creatinine by the method of Folin as adapted to the Coleman clinical spectrophotometer, and thiosulfate by the method of Gilman, Philips, and Koelle (9). The data indicate that, unlike inulin, creatinine, and thiosulfate, the clearance ratios of which approximate unity, mannitol is reabsorbed or destroyed in the kidney to the extent of about 10 per cent of that which is filtered. The data therefore confirm unpublished observations of Earle (personal communication) comparing mannitol-inulin clearance ratios and Hoobler (personal communication) as concerns mannitol-thiosulfate clearance ratio in human beings.

Clearances	Mean ratio	No of observations	Standard deviation	Standard deviation of mean
Mannitol inulin, dogs and human beings	0 902	42	±0 113	±0 017
Mannitol-creatinine, dogs	0 872	35	±0 079	±0 013
Mannitol-thiosulfate, dogs	0 89	12		
Inulin creatinine, dogs	0 98	12		
Inulin-thiosulfate	1 04	7		
Creatinine-thiosulfate	1 03	12		

simultaneous plasma mannitol, inulin, creatinine, and thiosulfate clearances is shown in Table II

### DISCUSSION

Any substance oxidized by periodic acid may interfere with the determination. The interference may arise either from the formation of formaldehyde from substances other than mannitol or from excessive consumption of periodic acid. Among the compounds that may interfere because of formaldehyde production are fructose,  $\alpha$ -glycerophosphate, ethylene and propylene glycol, and most compounds which contain either a  $\text{—CHOH—CH}_2\text{OH—}$  or a  $\text{—CO—CH}_2\text{OH—}$  grouping. Substances containing a  $\text{—CHOH—CHOH—}$  or a  $\text{—CO—CHOH—}$  or a  $\text{—CO—CO—}$  group are oxidized but do not yield formaldehyde. These may only interfere with the determination if they are present in such concentrations as will decrease the periodic acid content so that excess stannous chloride begins to reduce formaldehyde.

Of the interfering substances present in blood and urine the most important is glucose. In acid solution, glucose exists chiefly as the pyranose ring form which does not have a free  $\text{—CHOH—}$  group adjacent to the terminal  $\text{—CH}_2\text{OH—}$  group. Therefore, under the above conditions the terminal link in the carbon chain is very little oxidized in 10 minutes (formaldehyde production about 5 per cent of theoretical). It is possible therefore to determine mannitol accurately in the presence of small and constant concentrations of glucose by the above procedure. Thus, at concentrations of glucose of about 10 mg per cent in plasma filtrate, recovery of added mannitol averages 95 per cent and at glucose concentrations of 20 mg per cent it averages about 90 per cent. Several factors participate in this, of which the most obvious are (a) inequality of formaldehyde liberation from glucose as between mannitol-containing and mannitol-free samples, which is possibly due to (b) inequalities of periodic acid consumption and stannous chloride excess as between such samples. The difficulty may be largely obviated by addition of mannitol to the mannitol-free, glucose-rich plasma or urine samples and determination of unknowns from the curve thus established.

*Plasma Blank*—The normal plasma blank is equivalent to 3 to 5 mg of mannitol per 100 cc. About half of this blank is due to glucose. It therefore varies during wide fluctuations of the plasma glucose, but in the absence of these is constant in any person over periods of several hours.

*Urine Blank*—The average blank found in undiluted urine is about 0.3 to 0.5 mg per minute. We think it likely that the bulk of this urinary blank is due to the non-fermentable reducing substance normally found in urine.

*Time of Oxidation*—Under the conditions employed, oxidation of man-

nitrol is complete in 3 to 5 minutes While the reaction could be checked at this point, 10 minutes are chosen as a more convenient interval (Table III) If oxidation is prolonged beyond 10 minutes, the shifting of the

TABLE III  
*Relationship between Time of Oxidation, Temperature, and Optical Density in a 6-302 Cuvette*

Time	Temperature	Mannitol added	Optical density (D)	Deviation from 10 min value
<i>min</i>	<i>°C</i>	<i>mg per 100 cc</i>		<i>per cent</i>
10	9	1	0 191	0
20	9	1	0 191	0
30	9	1	0 191	0
40	9	1	0 191	0
60	9	1	0 193	+0 6
10	9	4	0 707	0
20	9	4	0 702	-0 7
30	9	4	0 702	-0 7
40	9	4	0 704	-0 4
60	9	4	0 707	0
4	26	1	0 192	-0 5
6	26	1	0 196	+2 1
8	26	1	0 192	-0 5
10	26	1	0 193	0
4	26	4	0 701	+0 7
6	26	4	0 701	+0 7
8	26	4	0 701	+0 7
10	26	4	0 696	0
Mean				±0 1

TABLE IV  
*Effect of Temperature on Optical Density in a No 6-302 Cuvette*

Mannitol concentration	$\Delta D$ at 20°	$\Delta D$ at 30°	$\frac{\Delta D 30^\circ}{\Delta D 20^\circ}$
<i>mg per 100 cc</i>			
0 5	0 100	0 108	1 08
2 0	0 360	0 398	1 10
4 0	0 672	0 732	1 09

equilibrium mixture of glucose isomers results in higher yields of formaldehyde from glucose

*Determination of Formaldehyde*—In applying MacFadyen's procedure under these conditions, we reinvestigated the effect of the time of heating

during color development and the effect of the temperature of the solution during the reading of the color

The time of heating of the formaldehyde-containing solutions was chosen as 30 minutes. At this time about 95 per cent of the maximum color has been developed, the full 100 per cent being reached only after 75 minutes.

The temperature of the solution should be adjusted to 25° with some care at the time of reading. There is about 9 per cent more color at 30° than at 20° (Table IV).

#### SUMMARY

A method is described for the determination of mannitol in plasma and urine at levels of 0.5 to 3.0 mg per 100 cc of filtrate or diluted urine. The method is based on the colorimetric estimation of the formaldehyde produced during controlled periodic acid oxidation of mannitol. Conditions are given for application of the method to determination of renal function under normal conditions.

The authors acknowledge the technical assistance of Mr R. Harris and Mr E. Balfour, the generous donation of mannitol solution by Dr K. Beyer and Dr W. Boger of Sharp and Dohme, Inc., and of chromotropic acid by Dr A. L. Fox and Mr Morgan Collins of the General Aniline and Film Corporation.

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# THE ISOLATION OF ESTRONE FROM THE BILE OF PREGNANT COWS\*

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(Received for publication, June 21, 1947)

Although estrogenic material has been demonstrated to be present in the bile of several mammalian species (1, 2), the chemical nature and quantity of the biliary estrogens of endogenous origin have not been determined. Such information might contribute to a better understanding of the rôle of the liver in the metabolism of estrogens and might have a bearing on a current theory (3) of an enterohepatic circulation of estrogens. The bile of cows in an advanced stage of pregnancy was deemed to be suitable experimental material for this study, inasmuch as the level of estrogen production during pregnancy is relatively high and because the bile of this species is readily obtainable in quantities adequate for isolation purposes.

Butanol extracts were made of an 80 liter and of a 230 liter pooled specimen (designated as Batches I and II) which had been previously assayed for estrogenic activity. Most of the estrogenic material in the butanol residues was removed by extraction with acetone, the acetone-insoluble material comprising the bulk of the organic material of the bile. The acetone-fractionated products were partitioned into (a) ether-soluble and (b) ether-insoluble, water-soluble moieties. The ether-soluble material was separated into acid, phenol, and neutral fractions. The phenols were partitioned into strongly acidic and weakly acidic phenols, the latter were fractionated into ketones and non-ketones. The ether-insoluble, water-soluble material of the butanol extracts was subjected to acid hydrolysis, followed by extraction with ether. The ethereal extract was partitioned into acid, phenol, and neutral fractions.

The estrogenic activity of the phenolic and acidic fractions was determined by bioassay on spayed adult mice by a vaginal smear technique (4). The results are summarized in Table I. Most of the estrogenic material in the bile of pregnant cows appears to be in an uncombined form, a finding which is similar to that reported for the exogenous estrogen of dog bile (3).

\* This work was supported by grants in-aid from the United States Public Health Service, under the National Cancer Institute Act, and from Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

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From the ketonic phenolic material derived from 31.0 liters of unhydrolyzed bile of pregnant cows, there were isolated 20 mg of a crystalline product, m p 251–254°. It was purified and its identity as estrone established by determination of melting point, mixed melting point with authentic es-

TABLE I  
*Distribution of Estrogen in Bile of Pregnant Cows\**

The results are given in mouse units

Volume of bile extracted	Batch No	Untreated bile†	Free phenols			Conjugated phenols (after hydrolysis)
			Weakly acidic		Strongly acidic	
			Ketonic	Non ketonic		
<i>liters</i>						
8 0	I	64,000	33,000	12,000	<1000	6600
23 0	II	620,000	120,000	54,000	<3300	6400
			Free acids			Conjugated acids (after hydrolysis)
8 0	I		<2000			<2000
31 0	I + II					

\* Bioassay was performed on spayed adult mice by use of a vaginal smear technique based on that described by Allen and Doisy (4). The following values were obtained when oily solutions containing 1  $\gamma$  of crystalline estrone,  $\alpha$ -estradiol, and estriol were injected subcutaneously 14, 33, and 0.66 mouse units respectively, when aqueous solutions of 1  $\gamma$  amounts of these estrogenic compounds were employed, the following values (in the same sequence) were obtained 22, 40, and 0.30 mouse units.

† Several factors may be responsible for the fact that the observed estrogenic activity of untreated bile is higher than the sum of the estrogenic activities of the fractionated bile extracts: (a) the vehicle employed for injection in the bioassay procedures was aqueous in the former instance but oily in the latter (*cf.* bioassay values cited above for the crystalline estrogens in both vehicles), (b) untreated bile contains a mixture of estrogens which were assayed against a standard solution of estrone, the ketonic and non ketonic extracts were standardized against estrone and  $\alpha$ -estradiol respectively, (c) the bile may contain unknown estrogenic substances which failed to be extracted, some loss of estrone may have been incurred during the course of extraction and fractionation, (d) experimental errors of bioassay.

trone, specific optical rotation, and by bioassay, a benzoyl derivative was prepared which gave the expected carbon and hydrogen values on analysis.

#### EXPERIMENTAL<sup>1</sup>

*Collection, Bioassay, and Extraction of Bile*—Gallbladder bile was freshly collected from slaughtered pregnant cows estimated to be at least 5 months

<sup>1</sup> All melting point values reported in this paper are corrected.

pregnant<sup>2</sup> The bile was refrigerated and worked up within 24 to 48 hours subsequent to collection. It retained a clear golden brown color at the time of extraction, the pH of the pooled specimens varied from 6.8 to 8.5. Bioassay of the untreated bile was performed by subcutaneous injection of the bile itself, or an aqueous dilution thereof, into spayed adult mice (4). There were approximately 6 to 41 mouse units of estrogene activity per ml of bile, the average value for eight pooled specimens was 22 mouse units per ml.

Prior to extraction, which was made with butanol with gentle shaking, the pH of the bile was adjusted to 6.5 to 7.5 with concentrated HCl. For every liter of bile, 500 ml of butanol were used in the initial extraction, followed by four extractions each with 100 ml of butanol. The extracts were combined, washed twice with 100 ml of water, and evaporated *in vacuo* to a syrupy consistency.

*Fractionation of Butanol Extracts*—Batch I (8.0 liters of bile) and Batch II (23.0 liters of bile) were worked up individually but in essentially identical fashion as in the following example. The syrupy butanol residue (Batch II) was slowly poured into 2 liters of acetone with stirring. The heavy precipitate (No. I) which formed was filtered and washed with a little acetone, the filtrate which contained most of the estrogene material was concentrated at atmospheric pressure and finally *in vacuo* to a syrupy consistency. The syrup was treated with 500 ml of acetone, whereupon an additional but smaller precipitate (No. II) was obtained. It was filtered and washed with a little acetone. The filtrate on evaporation yielded a residue (acetone-soluble fraction) which was taken up in 120 ml of warm alcohol and distributed between 450 ml of water and 3 liters of ether. The aqueous phase was combined with a solution of Precipitates I and II (acetone-insoluble fraction) in 1200 ml of water, made acid to Congo red with concentrated HCl, and extracted with 1500 ml and 300 ml of ether, the ethereal extracts were combined and washed well with water, whereupon beautiful white crystals appeared. Crystallization was allowed to proceed for a few days, about 4 gm of a nitrogenous acidic product, m p 184–185°, were thus obtained. It appeared to consist of conjugated bile acids from which desoxycholic acid was obtained on alkaline hydrolysis. The ethereal mother liquor was washed in several portions with 1200 ml of 4 per cent sodium bicarbonate (from which the organic acids were recovered on acidification and extraction with ether) and finally with water. The ethereal solution was combined with the ether extract of the acetone-soluble fraction.

<sup>2</sup> We are indebted to Dr. C. E. Mootz of the United States Department of Agriculture, Philadelphia, for arranging a veterinarian supervised collection of bile. Dr. Irwin Rothman was of great assistance in judging the approximate stage of pregnancy by examination of the fetuses.

(above) and evaporated. The residue (neutrals plus phenols) was partitioned between petroleum ether (b p 35–60°) and 90 per cent methanol. The 90 per cent methanol fraction was evaporated, the residue was dissolved in 200 ml of benzene and extracted four times each with 200 ml of *N* NaOH. The alkaline extracts were combined, back-washed with 40 ml of benzene, acidified, and thoroughly extracted with ether. The ether extract was washed with dilute  $\text{NaHCO}_3$  solution, then with water, and evaporated to yield the phenolic fraction (unhydrolyzed bile).

The aqueous solution of the acetone insoluble material mentioned above, following extraction with ether, was heated on the steam bath to remove dissolved ether. After the addition of 10 per cent by volume of concentrated HCl, the solution was refluxed for 10 minutes, cooled, and extracted with ether. The ethereal extract was fractionated into acids, neutrals, and phenols by methods similar to those already described.

*Fractionation of Phenols (Unhydrolyzed Bile)*—The separation of the phenols into benzene-soluble and sodium carbonate-soluble fractions is attended with great difficulty. It was necessary to employ the following fractionation scheme in order to attain constant values for the distribution of estrogenic activity. The phenols (Batches I and II individually) were repeatedly partitioned between benzene and 0.3 *M*  $\text{Na}_2\text{CO}_3$  according to the method of Mather (5). Troublesome emulsions resulted which were broken up by centrifugation. The alkaline extracts were acidified and extracted with ether. The ethereal residue was partitioned between petroleum ether and 90 per cent methanol. The aqueous methanol extract was evaporated and the residue partitioned between benzene and 0.3 *M*  $\text{Na}_2\text{CO}_3$ , the phenols were recovered from the latter phase as before. The benzene-soluble phenols were separated into ketonic and non-ketonic fractions with the aid of Girard's Reagent T (6). The estrogenic activity of the respective phenolic fractions is indicated in Table I.

*Isolation and Identification of Estrone*—Treatment of the ketonic phenols (160 mg in all) of the unhydrolyzed bile with a little alcohol yielded crude estrone. There were obtained from Batch I 4 mg of crude estrone, m p 251–252°, and from Batch II 16 mg, m p 252–254°. The crystalline products were combined and repeatedly crystallized from alcohol to give 3.0 mg, m p 256–258°,  $[\alpha]_D^{20} = +161^\circ$  (0.220 per cent in dioxan), 1  $\gamma \cong 10$  mouse units of estrogenic activity. The material used in the determination of optical rotation was recovered and crystallized once from alcohol, yielding 2.0 mg, m p 257.5–259°. It gave no depression in melting point on admixture with authentic estrone, m p 259–260°,  $[\alpha]_D^{20} = +163^\circ$  (in dioxan), 1  $\gamma \cong 14$  mouse units of estrogenic activity.

Acylation of 13.6 mg, m p 251–253°, of biliary estrone was effected by the Schotten-Baumann method by dissolving this product in 2 ml of *N*

NaOH, and treating with 5 drops of benzoyl chloride, with vigorous shaking after the addition of each drop. The white precipitate which formed was centrifuged, washed with a little  $N$  NaOH, and then washed well with water. The benzoyl derivative was repeatedly crystallized from chloroform-alcohol, yielding 7.4 mg,  $m.p.$  220–221°. It gave no depression in melting point on admixture with authentic estrone benzoate,  $m.p.$  222–223°.

$C_{25}H_{26}O_2$ . Calculated, C 80.18, H 7.00, found, C 80.30, H 6.94

#### DISCUSSION

The major estrogen of pregnant cow bile appears to be estrone which, on the basis of isolation, is present in amounts of approximately 600  $\gamma$  per liter of gallbladder bile. There may also be present as much as 70  $\gamma$  of  $\alpha$ -estradiol per liter on the assumption that the estrogenic activity of the non-ketonic, weakly acidic phenols is due principally to  $\alpha$ -estradiol. Since very little or no biological activity is exhibited by the strongly acidic phenols, the presence of estriol in the bile of this species appears to be precluded. While the total estrogen content of the bile seems to be low, it is considerable by comparison with estrogen levels in blood. Szego and Roberts (7) estimated that there are 3.4  $\gamma$  equivalents of  $\alpha$ -estradiol per liter of whole blood of cows 6 months pregnant. It would appear from the foregoing data that the liver, in this species at any rate, removes estrogen from the blood and concentrates it in the bile. This interpretation lends a measure of support to a theory of an enterohepatic circulation of estrogens under physiological conditions which was suggested by Cantarow *et al.* (3). In a previous report (2) by the latter authors, it was shown that the estrogen level in the bile<sup>3</sup> of women during pregnancy and post partum is higher than that in the blood.

Since the ratio of free to combined estrogen in the blood (7) is considerably lower than that in the bile, the question is raised as to whether the liver removes combined estrogen from the blood. If this is the case, is the liver capable of effecting hydrolysis of conjugated estrogen? The chemical nature and relative amounts of the estrogens of the blood of the pregnant cow (or of other species during pregnancy) are not known. Such information might be an aid in gauging the extent to which the liver may effect metabolic interconversions of the estrogens under physiological conditions.

It may be of interest to compare the data presented in this report with similar data obtained by Levin (8) on the estrogens in the feces of pregnant cows. The latter author indicated that most of the estrogenic activity of

<sup>3</sup> This refers to liver bile. Estrogen levels in gallbladder bile and in liver bile are not strictly comparable, since the former is considerably more concentrated.

the feces may be due to  $\alpha$ -estradiol, whereas our data indicate that estrone is the major estrogen of the bile. Biliary estrogen may be a source of fecal estrogen, as has been suggested by Levin (8), intestinal flora may be responsible for the apparent conversion of estrone into  $\alpha$ -estradiol. It is conceivable, however, that estrogen enters the bowel from the blood stream by passage through the intestinal wall in view of the recent finding (9) that significant amounts of estrogen appear in the feces of external bile-fistula dogs receiving estrone by subcutaneous injection. The data on the bile and feces of the pregnant cow are similar in that practically all of the estrone is present in the free form, and also in that there is little or occasionally no estrogenic activity in the strongly acidic phenolic material. The urinary excretion of estrogen by the pregnant cow (10) is relatively low compared to other species during pregnancy.

Miescher (11) recently made the interesting suggestion that estric acids of the doisyonic type might be normally present in the organism. Although demonstrable estrogenic activity has not been reported for the acid fractions of urine, it is conceivable that biologically active acids occur in the bile. Our data (see Table I) indicate, however, that very little or no estrogenic activity is exhibited by the small quantity of free acids occurring in unhydrolyzed bile or by the acids obtained on acid hydrolysis of the bile, inactive compounds of the marrianolic acid type may possibly be present in limited amounts.

#### SUMMARY

Estrone has been isolated from gallbladder bile of cows in an advanced stage of pregnancy in amounts approximating 600  $\gamma$  per liter, it appears to be the major estrogen of bile. As much as 70  $\gamma$  of  $\alpha$ -estradiol may be present in the non-ketonic, weakly acidic phenols, little or no estrogenic activity is exhibited by the strongly acidic phenols. Most of the estrogenic material of the bile is present in a free or uncombined form.

The implications of these findings with reference to the metabolism of the estrogens in the liver and in connection with a theory of an enterohepatic circulation of estrogens under physiological conditions are discussed.

The technical assistance of Miss Emily Cerceo, Miss Edith Goldberg, and Miss Dorothy Ozer is gratefully acknowledged. We are indebted to Mr James Rigas for the microanalysis.

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# THE VERATRINE ALKALOIDS

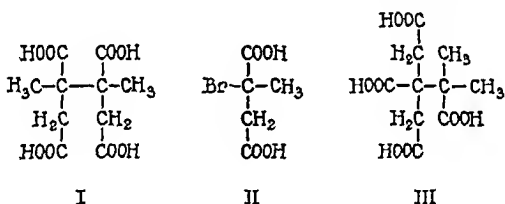
## XXVI ON THE HEXANETETRACARBOXYLIC ACID FROM CEVINE AND GERMINE

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(Received for publication, April 18, 1947)

From the mixture of acidic substances produced by chromic acid oxidation of cevine (1) and germine (2), an optically active hexanetetracarboxylic acid,  $C_{10}H_{14}O_8$ , was isolated, and certain data obtained in its study served as the basis for its provisional interpretation. The rate of hydrolysis of its tetramethyl ester was interpreted to indicate the presence of two tertiary carboxyl groups, since two of the ester groups were relatively resistant to alkaline hydrolysis (Fig 1, Curve 1). This observation together with the fact that the acid yielded a keto monoanhydride,  $C_9H_{10}O_4$ , on distillation led to its tentative interpretation as  $\beta, \beta'$ -dimethyl- $\beta, \beta'$ -



dicarboxyadipic acid (Formula I). A *meso* isomer as well as *d* and *l* stereoisomers is possible for this acid. The production of such an acid with two angular methyl groups, barring rearrangements, suggested a modified sterol ring system for cevine in which Ring B is 5-membered, and that the extra carbon atom appears as an angular methyl on carbon atom 5 (1).

To test the validity of this interpretation, a synthesis of  $\beta, \beta'$ -dimethyl- $\beta, \beta'$ -dicarboxyadipic acid became desirable and the results of this study are reported here. For this purpose citraconic anhydride was converted to *dl*- $\alpha$ -bromo- $\alpha$ -methylsuccinic acid (Formula II) according to Fittig and Landolt (3). Its methyl ester was condensed by heating with granular silver at 125–130° and yielded a syrupy mixture of esters. Both the *meso* form and a *dl* form of  $\beta, \beta'$ -dimethyl- $\beta, \beta'$ -dicarboxyadipic tetramethyl ester were to be expected. This reaction was suggested by the procedure already used by Hell (4) and by Auwers and Meyer (5) for the preparation of tetramethylsuccinic acid from  $\alpha$ -bromoisobutyric acid ethyl ester. However, Auwers and Meyer (6) obtained as a by-product  $\alpha$ -trimethylglutaric acid, which may have been the result of a rearrangement during

From an inspection of the saponification curves of the ester from cevine (Fig 1), it would appear that the third ester group to be saponified must be secondary in character because of its relative ease of saponification as compared with the tertiary ester groups in the synthetic esters. The natural acid may then contain two primary carboxyl groups, a secondary and possibly, although not certainly, a tertiary carboxyl group. Such an acid could arise from two points in the sterol structure, in which a tertiary carboxyl group is formed by the oxidation of a carbon atom attached to either carbon atom 10 or 13. If it can be established later from which portion of the molecule this acid really arises, certain positions will be excluded in locating the eight hydroxyl groups in cevine and germine.

As opportunity presents itself, studies will be continued to determine the identity of the tetracarboxylic acid obtained from cevine.

#### EXPERIMENTAL

*Meso-β,β'-Dimethyl-β,β'-dicarboxyadipic Tetramethyl Ester*—Citraconic anhydride (11) was converted to *dl-α-bromo-α-methylsuccinic acid* as described by Fittig and Landolt (3). The latter reported the melting point as 148°, whereas our substance melted at 142°. 15 gm of the acid suspended in 50 cc of ether were esterified at 0° with diazomethane. After removal of ether and excess reagent, the residue was fractionated at 13 mm. 12 gm of the dimethyl ester were collected at 121–125°. This ester was also prepared by refluxing for 2 days 15 gm of the bromo acid in 200 cc of methanol which contained 4 per cent HCl. 10 gm of the ester were collected at 120–125° and 13 mm. Both ester preparations yielded the same results when condensed as follows.

12 gm of the ester were heated with 15 gm of silver at 125–130° for 36 hours with occasional shaking. The silver used was prepared by reduction of an aqueous alkaline suspension of silver oxide with formaldehyde and, after successive washing with water and alcohol, was dried in a desiccator over P<sub>2</sub>O<sub>5</sub>. The above condensation mixture was filtered from silver salts which were washed repeatedly with ether. The filtrate was concentrated and the residue was distilled at 15 mm. After removal of a forerun at 90–92°, most of the product distilled at 140–145° and 0.3 mm. Partial crystallization of the syrup occurred on long standing. The material was collected with a minimal amount of ether and recrystallized from ethanol. 0.8 gm of flat needles was obtained, which melted at 128–130°.

C <sub>14</sub> H <sub>22</sub> O <sub>6</sub>	Calculated	C 52.80,	H 6.97,	OCH <sub>3</sub> 39.07,	mol wt 318.18
	Found	" 53.04,	" 6.48,	" 39.20,	" " 317

*meso-β,β'-Dimethyl-β,β'-dicarbomethoxyadipic Acid*—60.7 mg of the tetramethyl ester were heated at 100° with 6.5 cc of 0.157 N NaOH. 0.5 cc

samples were withdrawn at intervals for titration with 0.02 N acid. The course of hydrolysis is shown in Fig. 1 (Curve 2). This shows the prompt saponification of two carbomethoxy groups and relative resistance of the remaining two carbomethoxy groups. After continued heating, the solution was recombined with the titration samples, acidified with HCl, concentrated to a few cc., and then repeatedly extracted with ether. The latter yielded a residue which was recrystallized from ether. 45 mg. of the dimethyl ester which separated as heavy rhombs were collected (m.p. 210–215°).

$C_{12}H_{18}O_8$	Calculated	C 49.63,	H 6.25,	OCH <sub>3</sub> , 21.39
	Found	" 49.64,	" 6.11,	" 19.72

The following attempt was made to resolve this dimethyl ester. 1.5 gm. of the ester and 1.5 gm. of cinchonidine were dissolved in 10 cc. of ethanol. On cooling, the alkaloid salt crystallized. It was recrystallized three times from ethanol, then suspended in ether and shaken with 5 per cent NaOH. The alkaline phase was reacidified and the acid obtained by repeated extraction with ether melted at 210–215°. 150 mg. were thus recovered. A 7.4 per cent solution in methanol showed no appreciable rotation.

*dl-β,β'-Dimethyl-β,β'-dicarbomethoxyadipic Acid*—23 gm. of the syrup which remained after filtration of the above crystalline *meso* compound were saponified on the steam bath for 1½ hours with 300 cc. of 15 per cent NaOH. After acidification, the mixture was extracted five times with 50 cc. of ethyl acetate. 20 gm. of a syrup of mixed acids were obtained. A counter-current separation was run on the mixture at 25°. For this purpose nine separatory funnels were used, each of which contained 100 cc. of ethyl acetate and 100 cc. of a 2 M phosphate buffer (pH 5.1) as the phases. After the distribution, the contents of each funnel were acidified with 10 cc. of 40 per cent phosphoric acid, extracted with ethyl acetate, and separately investigated. The residue (2 gm.) from Funnel 2 was mostly crystalline and the residue (2 gm.) from Funnel 3 entirely crystalline. These fractions were combined and recrystallized from a mixture of ethyl acetate and light petroleum and then from water. 2.6 gm. of stout rhombs were obtained, which melted at 212–217°. The melting point of a mixture with the *meso*-dimethyl ester was 188–200°.

$C_{12}H_{18}O_8$	Calculated	C 49.63,	H 6.25,	neutralization equivalent 145.07
	Found	" 49.90,	" 6.29,	" " 147

*l-β,β'-Dimethyl-β,β'-dicarbomethoxyadipic Acid*—The salt prepared from 1 gm. of the *dl* form and 1 gm. of cinchonidine was recrystallized three times from ethanol. The free acid, regenerated as described above, weighed 240 mg. and melted at 225–228°.

$$[\alpha]_D^{25} = -33^\circ \quad (c = 7.9\% \text{ in methanol})$$

$C_{12}H_{18}O_8$	Calculated,	C 49.63,	H 6.25,	found, C 49.48,	H 6.18
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# THE ACONITE ALKALOIDS

## XVII FURTHER STUDIES WITH HETISINE

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(Received for publication, May 14, 1947)

In the course of the isolation of the alkaloids from the roots of *Aconitum heterophyllum* obtained in a more recent commercial shipment from India, it has been found that the base hetisine occurs in larger amounts than originally suspected. About 1 gm. per kilo has now been isolated.

The procedure used for the separation of the alkaloid fractions was essentially as previously described (1). But following the extraction of the strongly basic alkaloid fraction with benzene a continued extraction of the remaining aqueous phase with chloroform has now yielded a fraction which contains most of the hetisine and which could be readily isolated as the hydrochloride. To complete the extraction it was found necessary to continue the process for a number of days. The isolation of this material has made possible a more extensive study of the alkaloid which became especially desirable because of its relatively simple formulation of  $C_{20}H_{27}O_3N$ . Further analytical studies with the base and its derivatives have confirmed this formulation.

At first the attempt was made which was but partly successful to carry its degradation through the steps of exhaustive methylation. *Methyl-hetisinium iodide* was readily prepared, which was in turn converted to the methine base, *des-methylhetisine*. The latter and its *hydrochloride* were crystalline. The base with methyl iodide yielded a crystalline *methyl-des-methylhetisinium iodide* from which a *chloride* was also prepared. When, however, the attempt was made at the next stage of degradation to pyrolyze the ammonium hydroxide, methanol was split off instead of water with the production again of *des-methylhetisine*. This result was accompanied by the production of considerable unidentified amorphous material. However, the attempt to repeat the methylation of this amorphous fraction, followed by pyrolysis of its ammonium hydroxide, resulted again in almost quantitative recovery of basic material. The experiment was also unsuccessful at this stage for an Emde reduction to the methochloride.

As a modification of the procedure, *des-methylhetisine hydrochloride* was hydrogenated. At this point, instead of a tetrahydro derivative to be expected because of the original reactive double bond present in hetisine as well as that of the new methine bond, only 1 mole of  $H_2$  was absorbed with the production of a *dihydro-des-methylhetisine hydrochloride*. The free

base  $C_{21}H_{31}O_3N$  could not be crystallized. It seems probable, from the experience with dihydrohetisine discussed below, that the new methine bond must be the one hydrogenated and that in some way the original hetisine double bond has lost its reactivity. The free base yielded an apparent mixture of possibly isomeric crystalline derivatives with methyl iodide, each of which analyzed as a *dihydro-des-methylhetisininum methiodide*. Here again pyrolysis of the hydroxide resulted in loss of methanol with reformation of dihydro-des-methylhetisine.

Of interest was the behavior of dihydro-des-methylhetisine on oxidation with permanganate. It was smoothly converted to a base,  $C_{21}H_{29}O_3N$ , which readily crystallized and proved to be different from and apparently isomeric with des-methylhetisine. It is hoped that we can return to its study at a later time.

An attempt was made to apply the Hofmann degradation to dihydrohetisine as a starting point. However, a difficulty was at once encountered in the failure to obtain crystalline substances. At this point it may be mentioned that dihydrohetisine, previously described only as the hydrochloride, has since been obtained as the crystalline base. *Methyldihydrohetisininum iodide* was obtained only as a resin. Nevertheless, the free hydroxide was pyrolyzed to yield *des-methyldihydrohetisine*. The resinous base gradually crystallized on standing, but no satisfactory method for its recrystallization was found. On hydrogenation as the HCl salt, it absorbed 1 mole of  $H_2$  to form an amorphous *dihydro-des-methyldihydrohetisine*. Since the original double bond of hetisine had been disposed of, it appears that the new methine double bond must have been the point of hydrogenation. The hydrogenated base gave in turn a resinous methiodide. However, on pyrolysis of the ammonium base, it lost methanol as shown by the N-methyl content of the sublimed material.

A preliminary study of the dehydrogenation of hetisine with selenium has also been made. A tarry reaction product was obtained which could be separated into neutral and basic fractions. From the neutral fraction, by chromatographing through  $Al_2O_3$  and sublimation, it was possible to isolate several hydrocarbon fractions. One of these proved of significance, since it yielded, although in very small amount, a fraction which was finally crystallized as the trimnitrobenzene compound. The hydrocarbon recovered from the latter was found to be pimanthrene, as shown by analysis, absorption spectra (Fig. 1), and by comparison of its properties and those of the trimnitrobenzene compound with a sample of pimanthrene already isolated from staphisine (2). As in the case of the latter, the possibility of a partly hydrogenated diterpenoid structure is therefore suggested in the make-up of hetisine.

The major part of the hydrocarbon fraction from hetisine was a non

fluorescent material with a comparatively high hydrogen content. It could be separated from the fluorescent phenanthrene hydrocarbons with  $\text{Al}_2\text{O}_3$ . The molecular weight and analytical data indicated  $\text{C}_{34}\text{H}_{60}$ , or a system of six rings, as the formulation of the material. Since the fraction was a liquid and available in too small amount for fractional distillation, there could be no assurance of its homogeneity. The notable fact is, however, that a fraction of such comparatively high molecular weight and high hydrogen content is produced. Although this might suggest a pos-

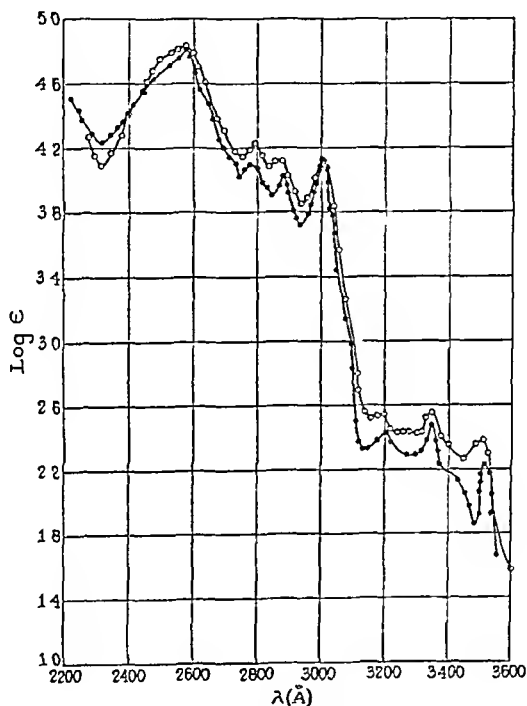


FIG 1 O, pimanthrene from hetisine, ●, pimanthrene from staphisine

sible double molecule for hetisine of  $\text{C}_{40}\text{H}_{54}\text{O}_6\text{N}_2$ , such a hydrocarbon could result from the condensation of smaller fragments during the dehydrogenation. This was shown by the determination of the molecular weight of hetisine. Because of its sparing solubility in camphor as originally reported, the Barger method has since been used which verified the simpler molecule of  $\text{C}_{20}\text{H}_{27}\text{O}_3\text{N}$ .

At this point it may be reported that the failure of hetisine to consume significant amounts of lead tetraacetate or sodium periodate are against the presence in it of reactive vicinal hydroxyl groups.



The basic fraction from the dehydrogenation was similarly separated with alumina into a number of fractions and at least partly purified by recrystallization of the picrates

Three fractions were obtained, one which on analysis gave  $C_{18}H_{17}N \cdot C_6H_3O_7N_3$ , a second either as  $C_{18}H_{17}N \cdot C_6H_3O_7N_3$  or  $C_{18}H_{15}N \cdot C_6H_3O_7N_3$ , and a third as  $C_{19}H_{23}ON \cdot C_6H_3O_7N_3$ . The first and second fractions may be identical. These substances were produced in very small amounts and, since the free bases themselves could not be crystallized, any attempt to interpret their significance would be premature.

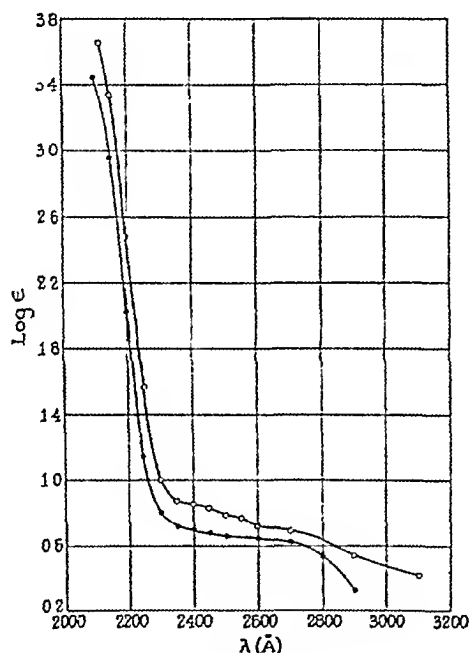


FIG. 2 O, hetisine in ethanol, ●, hetisine in ethanol with excess HCl

The ultraviolet absorption spectra of hetisine and its hydrochloride taken in alcoholic solution (Fig. 2) were found to show much less difference than in the case of atisine and heteratisine. The absorption as seen in Fig. 2 is mostly end-absorption of an unspecific type which does not permit of obvious conclusions.

Since the nitrogen atom of hetisine is tertiary and apparently common to two rings, the additional three rings of the perhydrophenanthrene portion would require a minimum of five rings for the alkaloid. Although no crystalline O-acyl derivatives of the latter have been obtained, the active hydrogen determination has indicated the presence of three OH groups (1).

One double bond has been shown by hydrogenation. From the formulation  $C_{20}H_{27}O_3N$  for the base, there remain therefore two undetermined points of unsaturation. Whether these consist of resistant double bonds or rings remains a problem.

#### EXPERIMENTAL

The rhizomes of *Aconitum heterophyllum* used in the following studies were obtained from an Indian source through S B Penick and Company.

The procedure for the extraction of the ground material was essentially as previously described, with a few changes. Each 8 kilo lot was extracted for a day with 24 liters of 70 per cent alcohol and then by three succeeding daily treatments with 16 liters of solvent. After concentration of the extract to a thin aqueous syrup and acidification with 10 per cent  $H_2SO_4$ , the mixture was extracted four times with a total of 2 liters of chloroform, which in turn was washed several times with small portions of water. The combined aqueous fractions were carefully neutralized with  $Na_2CO_3$  solution and extracted with three 1 liter portions of benzene. This benzene extract of the weaker bases was washed several times with minimal amounts of water and kept separate. The combined aqueous phase was then emulsified with 1500 cc of benzene and rapidly made strongly alkaline with 200 cc of 25 per cent NaOH and well shaken. The extraction was repeated six times with 500 cc portions of benzene. After washing with several small portions of water the dried extract was concentrated *in vacuo* to a resin of bases which were in turn converted as previously described into the HCl salts in absolute alcoholic solution. Successive fractions of crystalline salts were obtained which totaled about 40 gm from each 8 kilos and consisted mainly of atisine hydrochloride, although the final fractions yielded small amounts of heteratisine and hetisine hydrochloride.

Following the above extraction with benzene, the alkaline aqueous phase (about 4 liters) was extracted consecutively in several funnels six times with a total of about 1500 cc of chloroform. The washed and dried extract yielded a resinous residue which was converted in alcoholic solution to the HCl salt. The latter crystallized copiously and after collection weighed about 4.3 gm. At this point the amount varied in different experiments, the variation being possibly due to differences in the extraction conditions. Such differences, however, were as a rule compensated for by the amount obtained in the succeeding continuous extraction.

$[\alpha]_D^{25} = +12.5^\circ$ ( $C = 1.01$ in 95% ethanol)	
$C_{20}H_{27}O_3N$ HCl	Calculated C 65.63, H 7.72
	Found (a) " 65.48, " 7.61
	(b) " 65.10, " 7.42

Under the microscope the substance changed above  $320^{\circ}$  and gradually lost its double refraction at  $328-335^{\circ}$

For comparison, a sample of hetisine hydrochloride was prepared from the hetisine previously described (1). Under the microscope it began to change above  $320^{\circ}$  and gradually lost its double refraction at  $330-340^{\circ}$

$$[\alpha]_D^{25} = +12.7^{\circ} \text{ (c = 1.06 in 95\% ethanol)}$$

Found, C 65.80, H 7.72

At this stage it was found advisable to continue the extraction process in a continuous liquid extractor for 5 or 6 days. After each day the boiling chloroform solution of extracted material was replaced by fresh solvent, since an apparent condensation of chloroform with substances in the alkaline aqueous phase caused formation of obscure resinous products which made purification of the alkaloid more difficult. The basic material recovered from the combined chloroform extracts was converted in alcoholic solution to the HCl salt. 3.8 gm. more of hetisine hydrochloride were thus obtained.

Found, (a) C 65.45, H 7.72, (b) C 65.23, H 7.45

The total amount of crude hetisine hydrochloride recovered from approximately 119 kilos of ground aconite root was about 112 gm.

The base prepared from the hydrochloride separated gradually from dilute alcohol as glistening stout prisms which contained solvent and effervesced at  $145^{\circ}$ . The melting point varied with the conditions of crystallization. For analysis it was dried at  $100^{\circ}$  and 0.2 mm.

$C_{20}H_{17}O_3N$  Calculated, C 72.90, H 8.27, found, C 72.90, H 8.03

Hetisine was found to sublime almost completely at 0.1 mm. from a bath at  $190-210^{\circ}$ . If the material used contained a solvent, a small amount flashed over even at  $145-160^{\circ}$ .

Found, C 72.89, H 8.23

A solution of 41.2 mg. of hetisine in 1 cc. of ethanol was found in equilibrium with a solution of 42.0 mg. of isoetisine (mol. wt. 343.3) in 1 cc. of ethanol, as determined by the Baizer method.

$C_{20}H_{17}O_3N$  Calculated, mol. wt., 329.3, found, 336

*Dihydrohetisine*—Instead of the procedure originally reported, the conditions used by Sugimoto and Shimanouchi (3) for the hydrogenation of kobusine were repeated here in an unsuccessful effort to carry the hydrogenation beyond the dihydro stage.

0.1 gm. of alkaloid dissolved in acetic acid was hydrogenated with 50 mg. of platinum oxide catalyst. After the prompt absorption of approxi-

mately 1 mole beyond the catalyst requirement, there was no significant further absorption, even after addition of more catalyst. After 1 week the process was discontinued, the filtrate from the catalyst was concentrated, the base was liberated in aqueous solution with alkali, and then extracted with chloroform. A resin was obtained on concentration which was dissolved in a small volume of methanol. After addition of water the solution was boiled down to 3 cc to remove methanol. On seeding, dihydrohetisine crystallized slowly and incompletely as pointed microprisms which contained solvent. Under the microscope it softened to a resin at 136–139° which gradually crystallized again on further heating. The apparently anhydrous material then partly softened, especially above 220°, and melted at 250–255°.

For analysis, the substance was dried at 100° and 0.2 mm.

$C_{20}H_{29}O_3N$  Calculated, C 72.45, H 8.82, found, C 72.27, H 8.77

*Methylhetisine Iodide*—0.3 gm of hetisine was heated with 1 cc of methyl iodide in a sealed tube at 100° for 2 hours. The partly crystalline mass was boiled down to remove the excess reagent and treated with methanol. After concentration to smaller volume and addition of ether, the reaction product crystallized as needles or rods. 0.19 gm was collected in a first fraction. Under the microscope the substance, after preliminary sintering above 300°, did not lose its double fraction till 320–325°.

$C_{21}H_{30}O_3NI$  Calculated C 53.48, H 6.42, I 26.94  
Found " 53.28, " 6.26, " 26.94

The mother liquor on further addition of ether gave a second fraction of 0.12 gm.

Found, C 53.22, H 6.28, I 26.32

*Des-methylhetisine*—2.5 gm of the methiodide were dissolved in a small volume of methanol and decomposed with a slight excess of a suspension in methanol of freshly prepared  $Ag_2O$ . The filtrate was concentrated in the sublimation apparatus under reduced pressure and the residual resinous mass was gradually heated to 100° under the water pump as long as bubbling of the resin (due to loss of solvent and perhaps water) continued. The pressure was reduced with an oil pump to 0.3 to 0.4 mm and the heating continued with progressive temperature elevation to about 160° in 20 minutes. Although some condensation on the condenser became apparent above 135°, possibly due to continued  $H_2O$  cleavage the sublimation became especially profuse and rapid from 170–180°. In the course of an additional 20 minutes the bath was raised to 200° and the operation then interrupted. The practically colorless resinous sublimate was

washed off with methanol and again freed from solvent. The yield was 1.55 gm. This material gradually crystallized on long standing, but the base was preferably prepared from the hydrochloride described below by addition of excess of ammonia to its aqueous solution. The base separated as sparingly soluble flat needles which showed a micro melting point of 122–124° after slight preliminary softening.

For analysis it was dried at 100° and 0.2 mm.

$C_{21}H_{29}O_2N$  Calculated, C 73.42, H 8.52, found, C 73.30, H 8.55

The hydrochloride crystallized from methanol-ether as needles which melted under the microscope at 303–305° after softening above 297°.

$C_{21}H_{29}O_2NCl$  Calculated C 66.37, H 7.96, Cl 9.34  
Found " 66.20, " 7.70, " 9.24

*Methyl-des-methylhetisinium Iodide*—1.55 gm of the previous des base were heated with 8 cc of methyl iodide for 3 hours. The initial clear solution deposited a resin which crystallized as arborescent masses of prisms. After removal of the excess reagent, the mass was crystallized from chilled methanol as colorless prisms. The first two fractions (a and b) amounted to 1 gm.

$C_{22}H_{31}O_2NI$  Calculated C 54.41, H 6.65, I 26.16, N(CH<sub>3</sub>) 6.18  
Found (a) " 54.42, " 6.55  
" (b) " 54.56, " 6.49, N(CH<sub>3</sub>) 6.10, 5.90  
" " 54.79, " 6.68, " 25.78

After recrystallization from methanol, it sintered under the microscope above 237° and gradually softened to a melt at 246–250°.

When the attempt was made to recover material in the original mother liquor by the addition of ether, the obvious peroxide content of the latter caused liberation of iodine with crystallization of further fractions, but these were found to be high in iodine, apparently due to the addition of the latter to a double bond. This material was not further studied.

*Chloride*—The chloride was obtained from the iodide in methanol with AgCl. It crystallized from methanol-ether as flat needles which gradually melted under the microscope at 285–290°.

$C_{21}H_{29}O_2NCl$  Calculated C 67.05, H 8.19, Cl 9.01  
Found " 67.04, " 7.95, " 8.80

The attempt was made to carry the methiodide through the next stage of degradation. 0.15 gm of methiodide was converted with Ag<sub>2</sub>O in methanol to the hydroxide. After removal of solvent, the resinous residue was slowly heated in a sublimation apparatus at 0.2 to 0.3 mm. The bath during 20 minutes gradually reached 250–255° and was kept at this point 10 minutes more. The sublimate was washed off with methanol and after

removal of the solvent weighed 0.11 gm. In methanol it yielded a hydrochloride (46 mg) which separated as delicate needles on addition of ether. Analysis, however, showed the loss of a  $\text{CH}_3$  group and reconversion to *des*-methylhetisine hydrochloride.

$\text{C}_{21}\text{H}_{30}\text{O}_3\text{NCl}$	Calculated	C 66.37, H 7.96, N( $\text{CH}_3$ ) 3.95
	Found (a)	" 66.77, " 7.93, " 4.44
	(b)	" 66.25, " 7.78, " 4.30

Since again in a second experiment, but with a larger amount, only a fraction of the sublimed base crystallized as the hydrochloride (0.2 gm from 0.53 gm of sublimed base), the attempt was made to carry the dissolved material through the final step of degradation if still a dimethyl derivative. After removal of  $\text{Cl}^-$  in methanol with  $\text{Ag}_2\text{O}$ , during which an odor of trimethylamine became apparent, the dissolved base was freed from solvent and sublimed as usual. 0.33 gm of resinous sublimate was obtained which was found to be still entirely basic. The material could not be crystallized.

*Dihydro-des-methylhetisine*—1.42 gm of *des*-N-methylhetisine hydrochloride were hydrogenated in methanol with 0.1 gm of  $\text{PtO}_2$  catalyst. After 3 hours the reaction appeared complete when 87 cc or 1 mole of  $\text{H}_2$  had been absorbed by the substance.

The product separated in a volume brought to 5 cc as prismatic needles. 0.83 gm was collected at  $0^\circ$  with methanol. Under the microscope it began to change above  $300^\circ$  but especially at  $308$ – $310^\circ$ , and then disappeared by apparent sublimation from the hot stage at  $315$ – $318^\circ$ . This general behavior, the point of which varied somewhat with different samples or fractions, was not altered by recrystallization from methanol.

$\text{C}_{17}\text{H}_{22}\text{O}_3\text{NCl}$	Calculated, C 66.02, H 8.45, found, C 66.10, H 8.25
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Additional fractions were obtained by addition of ether to the mother liquor.

The free base could not be obtained crystalline.

An attempt to increase the absorption of  $\text{H}_2$  in the presence of an excess of  $\text{HCl}$  in methanol solution did not change the result.

Found, C 65.80, H 8.41
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*Dihydro-des-methylhetisine Methiodide*—The resinous base obtained from 0.22 gm of the above salt was heated with 2 cc of methyl iodide at  $100^\circ$  for 6 hours. An appreciable crystalline deposit formed which was separated from the solvent. The crystals on recrystallization from methanol yielded 95 mg of compact prisms or thin almost square platelets which melted under the microscope at  $249$ – $251^\circ$  after slight preliminary softening.

$\text{C}_{18}\text{H}_{24}\text{O}_3\text{NI}$	Calculated	C 54.19, H 7.03, N( $\text{CH}_3$ ) 6.16
	Found	" 54.47, " 7.14, " 6.50

The above methyl iodide mother liquor concentration yielded an appreciable resinous residue which crystallized from 1 cc of methanol at 0° as flat needles or platelets. This fraction (46 mg) sintered gradually above 263° and after some progressive softening melted incompletely from 273–280°, but crystals persisted till 295°.

Found, C 53.80, H 6.77, N(CH<sub>3</sub>) 6.98

The attempt to carry this methiodide through the next stage of degradation was as follows. 73 mg of methiodide (first fraction) after decomposition with Ag<sub>2</sub>O in methanol were freed from solvent and then sublimed at 0.2 mm. After heating to 215° the process was interrupted. The resin recovered from the condenser weighed 53 mg. After conversion to the HCl salt, 22 mg were obtained in a first fraction from methanol-ether. This material under the microscope gradually sublimed with decomposition at 296–300°.

C<sub>21</sub>H<sub>32</sub>O<sub>3</sub>NCl    Calculated    C 66.02, H 8.45, N(CH<sub>3</sub>) 3.93  
                          Found        " 65.93, " 8.42, " 4.33

*Oxidation of Dihydro-des-methylhetisine*—0.1 gm of the hydrochloride of this base in methanol was decomposed with a slight excess of silver oxide. After concentration of the clear filtrate all attempts to crystallize the free base were futile. The resin was dissolved in 10 cc of acetone and 0.1 cc of acetic acid. On addition of small amounts of powdered potassium permanganate, a gradual but definite reaction occurred, but then reached a point where the reagent was very slowly used up and the amount added had totaled about 25 to 30 mg. The filtrate was concentrated to remove the solvent and, after addition of water, the base was liberated with alkali and extracted with chloroform. The latter yielded on concentration 80 mg of a colorless resin which gradually crystallized. The base crystallized from a chilled concentrated methanol solution as well formed long needles or flat prisms. It melted under the microscope at 139–141° after slight preliminary sintering.

C<sub>11</sub>H<sub>19</sub>O<sub>3</sub>N    Calculated    C 73.42, H 8.52  
                          Found    (a) " 73.20, " 8.24  
    (b) " 73.34, " 8.22

*Dehydrogenation of Hetisine*—The resinous base obtained from 6 gm of hetisine hydrochloride by decomposition with NaOH and extraction with chloroform was mixed with 15 gm of selenium and heated in a slow stream of CO<sub>2</sub> for 2.5 hours at 340°. The amount of substance which distilled was inappreciable and was therefore discarded. The reaction mass was thoroughly extracted with hot benzene. This extract was shaken first with 10 per cent HCl and then with 5 per cent NaOH. The acid extract

was made alkaline and extracted with ether 0.75 gm of a crude basic fraction was thus obtained (Fraction B). The NaOH extract was found to contain no appreciable phenolic or acidic products. The benzene solution which remained and contained the neutral fraction yielded 2.25 gm of a brown tar on evaporation (Fraction N). Fraction B (0.75 gm) of crude bases was dissolved in benzene and passed through a 12 mm  $\times$  10 cm column of  $\text{Al}_2\text{O}_3$  (E. Merck and Company). On development with benzene three zones were noted, a lower zone which was distinguished by a blue fluorescence in ultraviolet light, an intermediate yellow zone, and an upper dark brown zone. The three zones were successively eluted with benzene. Fraction 1 amounted to 35 mg, Fraction 2 was 130 mg, and Fraction 3 was 235 mg. None of these could be crystallized directly.

On addition of an equal amount of picric acid in ethanol to Fraction 1 it crystallized copiously. After two recrystallizations from ethanol, 15 mg of small yellow platelets were obtained, micro mp  $225\text{--}230^\circ$  with decomposition.

$\text{C}_{18}\text{H}_{17}\text{N}$   $\text{C}_6\text{H}_3\text{O}_7\text{N}_3$  Calculated, C 60.48, H 4.23, found, C 60.44, H 4.39

Fraction 2 (130 mg) was rechromatographed on  $\text{Al}_2\text{O}_3$  from petroleum ether solution. By development with 40 per cent benzene in petroleum ether, a lower blue fluorescing band, followed in the ultraviolet light, was eluted from the column. 30 mg of an oil resulted, from which a crystalline picrate was readily prepared. On recrystallization from acetone, 28 mg of yellow-orange platelets were obtained, micro mp,  $235\text{--}245^\circ$  with decomposition.

$\text{C}_{18}\text{H}_{17}\text{N}$ $\text{C}_6\text{H}_3\text{O}_7\text{N}_3$	Calculated	C 60.48, H 4.23
$\text{C}_{18}\text{H}_{15}\text{N}$ $\text{C}_6\text{H}_3\text{O}_7\text{N}_3$	"	" 60.73, " 3.84
	Found	" 60.67, " 3.99

A mixture of this picrate with the picrate prepared from Fraction 1 melted at  $225\text{--}235^\circ$  with decomposition.

Fraction 3 yielded in similar manner a partially crystalline picrate which after two recrystallizations from acetone gave 10 mg of a light yellow powder, micro mp  $320\text{--}325^\circ$ .

$\text{C}_{19}\text{H}_{15}\text{ON}$ $\text{C}_6\text{H}_3\text{O}_7\text{N}_3$	Calculated	C 58.54, H 5.52, N 10.93
$\text{C}_{19}\text{H}_{17}\text{ON}$ $\text{C}_6\text{H}_3\text{O}_7\text{N}_3$	"	" 58.31, " 5.89, " 10.88
	Found	" 58.46, " 5.65, " 10.51

The neutral fraction (2.25 gm) was chromatographed from benzene on a 12 mm  $\times$  10 cm column of  $\text{Al}_2\text{O}_3$ . It was developed with benzene till the lower, blue fluorescing band (in ultraviolet light) was removed. 0.42 gm of a mobile yellow oil was obtained in this fraction. A second yellow zone was then eluted with benzene to yield 0.32 gm of a resin. The



uppermost dark brown zone was finally eluted with 1 per cent methanol in benzene and yielded 1.36 gm of a brown tar. These last two fractions were not further studied. The first fraction was rechromatographed on alumina from petroleum ether and then developed with a mixture of 10 per cent benzene in petroleum ether. The eluate, collected before the fluorescent zone had begun to emerge, yielded 180 mg of a colorless oil. The latter was distilled twice in a sublimation apparatus at 130° and 0.1 mm.

$C_{31}H_{58}$	Calculated	C 86.87, H 13.13, mol wt	446.45
$C_{31}H_{60}$	"	" 86.47, " 13.53, " "	448.47
	Found	" 86.70, " 13.40, " "	445, 450

The micro boiling point determined at 760 mm indicated it to be above 260°, and at 26 mm at about 185°.  $n_D^{26} = 1.4805$

The succeeding blue fluorescent zone, on elution with 30 per cent benzene in petroleum ether, yielded 20 mg of an oil. On addition of an equal weight of trinitrobenzene in acetone to this fraction and concentration to dryness, a residue was obtained which crystallized from ethanol as yellow needles, micro m p 156–159°.

$C_{16}H_{14}$	$C_6H_3O_6N_3$	Calculated	C 62.98, H 4.09
$C_{17}H_{16}$	$C_6H_3O_6N_3$	"	" 63.71, " 4.42
		Found	" 63.78, " 4.48

By chromatographing this substance through  $Al_2O_3$  from benzene, the hydrocarbon was separated from the trinitrobenzene. The oil obtained crystallized from ethanol and after recrystallization from ethanol, 4 mg of plates were obtained, micro m p 77–80°.

$C_{16}H_{14}$  Calculated, C 93.15, H 6.85, found, C 93.10, H 6.89

The melting point of a mixture of pimanthrene obtained by the dehydrogenation of staphisine (m p 80–84°) with this hydrocarbon was 80–84°. The trinitrobenzene derivative again prepared from the crystalline  $C_{16}H_{14}$  hydrocarbon formed yellow needles from ethanol which melted at 166–168°. The melting point of the trinitrobenzene derivative prepared from staphisine-pimanthrene was 166–168° and the melting point of a mixture of the two derivatives showed no depression. A mixture of the pimanthrene derivative with the above crude trinitrobenzene derivative having a melting point of 156–159° melted at 162–168°. It is most probable that this substance was an impure pimanthrene compound, as also indicated by the analysis. The melting point of the recrystallized complex of pimanthrene and trinitrobenzene, determined in a capillary, was 161.5–162°. Ruzicka and Sternbach (4) have reported the melting point as 158–160.5°.

All analytical work was performed by Mr. D. Rigakos.

## SUMMARY

An improved method for the isolation of hetisine has been found which involves the continuous extraction of a strongly alkaline aqueous solution with chloroform after preliminary extraction of the other alkaloids of the crude mixture with benzene. Degradation of hetisine by exhaustive methylation proceeded one step to des-methylhetisine but the latter was regenerated by pyrolysis of its quaternary ammonium hydroxide. Des-methylhetisine was hydrogenated to a dihydro derivative whose quaternary ammonium hydroxide also lost methanol to regenerate the original dihydro derivative. The Hofmann degradation applied to dihydrohetisine was also unsuccessful. In this case no crystalline intermediates could be isolated. Selenium dehydrogenation of hetisine yielded the hydrocarbon, pimanthrene, and very small amounts of three basic substances,  $C_{18}H_{17}N$ ,  $C_{18}H_{16}N$  or  $C_{18}H_{17}N$ , and  $C_{19}H_{25}ON$ . The latter were isolated as picrates.

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# THE ACONITE ALKALOIDS

## XVIII THE SYNTHESIS OF THE HYDROCARBON OBTAINED ON DEHYDROGENATION OF ATISINE

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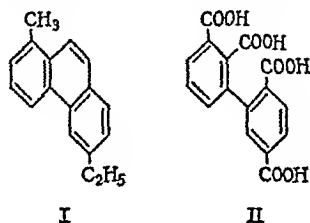
(Received for publication, May 23, 1947)

On the basis of the interpretation of the products obtained by its oxidation and of its physical properties, it appeared that the main phenanthrene hydrocarbon,  $C_{17}H_{16}$ , obtained from atisine on dehydrogenation could be either 1-methyl-6-ethylphenanthrene (Formula I) or 1-ethyl-6-methylphenanthrene (1). Since neither of these substances has been previously recorded, it was of importance to check the correctness of either possibility by synthesis. The synthesis of *1-methyl-6-ethylphenanthrene* has now been accomplished and the comparison of the hydrocarbon and its *picric acid* and *trinitrobenzene derivative* has shown them to be indistinguishable from the atisine hydrocarbon and its derivatives.

The Bogert and Cook method (2) has been used for the synthesis of 1-methyl-6-isopropylphenanthrene by Slater (3) and was closely followed in the present case.  $\beta$ (*p*-Ethylphenyl)ethyl bromide was prepared by the action of phosphorus tribromide on  $\beta$ (*p*-ethylphenyl)ethyl alcohol. The latter was obtained by sodium reduction of *p*-ethylphenylacetic ester but was used without purification for the preparation of the above bromide. The Grignard reagent prepared from the latter was condensed with 2,6-dimethylcyclohexanone (4). The resulting *carbinol* also without purification was cyclized with  $H_2SO_4$  and the crude octahydrophenanthrene derivative which was obtained was dehydrogenated with selenium. The resulting 1-methyl-6-ethylphenanthrene was separated from the mixture with aluminum oxide and isolated as the trinitrobenzene derivative (m.p. 148–149°). The picric acid melted at 134–136°. The hydrocarbon recovered from the picric acid melted at 43–44°. In melting points, mixed melting points, and other properties, the synthetic substances were indistinguishable from the  $C_{17}H_{16}$  hydrocarbon and derivatives from atisine.

As a further check on the 1,6 positions of the alkyl substituents in the  $C_{17}H_{16}$  hydrocarbon from atisine, a synthesis was attempted of the diphenyltetra-carboxylic acid which had been previously obtained from this hydrocarbon by oxidation. An unambiguous method for this purpose appeared to be the oxidation of 1,6-dimethylphenanthrene. The latter has already been described by Haworth, Marvin, and Sheldrick (5). Instead

of the method used by these workers, a somewhat different procedure has been employed paralleling the above preparation of 1-methyl-6-ethylphenanthrene  $\beta(p\text{-Tolyl})$ ethyl bromide was condensed with 2,6-dimethylcyclohexanone to yield the carbinol which in crude form was cyclized to the octahydrodimethylphenanthrene. The latter also without purification was dehydrogenated to 1,6-dimethylphenanthrene, which agreed in all properties with a sample kindly sent to us by Professor R. D. Haworth.



The quinone already described by Haworth, Mavin, and Sheldrick was prepared from the hydrocarbon and on oxidation with permanganate yielded *diphenyl-2,3,2',5'-tetracarboxylic acid* (Formula II). This acid and its *tetramethyl ester* were indistinguishable from the acid and ester obtained from atisine.

In a later communication will be described the conversion of an oxidation product of isoatisine to 1,6-dimethylphenanthrene, a result which therefore gives additional support to the present results. The meaning of the production of a 6-ethylphenanthrene on dehydrogenation is not clear in connection with the data at hand. It raises the question whether the suggested diterpenoid structure of atisine is modified by substitution in the 6 position or whether such substitution is contributed by the so-called N-ethyl group of the base. The usual 7-isopropyl group, if present, may be so modified as to be removed completely during dehydrogenation and so to escape detection. In the case of the  $C_{19}H_{26}$  hydrocarbon from staphisine its identification as 1,3-dimethyl-7-isopropylphenanthrene (6) has shown the occurrence of the usual 7-isopropyl group in this alkaloid. It is hoped to ascertain the significance of the production of a 6-ethylphenanthrene in other ways.

#### EXPERIMENTAL

*$\beta(p\text{-Ethylphenyl})$ ethyl Bromide*—*p*-Ethylphenylacetic acid was prepared according to Baker, Dippy, and Page (7), m.p. 90–92°. These authors reported a melting point of 92°. 12 gm. of the acid were refluxed for 24 hours in 200 cc. of 3 per cent HCl in ethanol. After removal of alcohol *in vacuo* and addition of water, the ester was extracted with ether. Fractionation yielded 11 gm. of ester, which distilled at 128–130° and 13 mm. The ester was reduced to the carbinol by the general procedure described

in "Organic syntheses" (8) For this purpose, 85 gm of sodium were disintegrated to a powder under 25 cc of toluene and 11 gm of the ester in 20 cc of absolute ethanol (dried by the magnesium methoxide method) were added in two portions The violent reaction soon subsided and 50 cc of ethanol followed by 10 cc of water were gradually added The toluene and ethanol were then removed with steam and the residual crude alcohol was extracted with ether The dried extract on concentration yielded 8 gm of the alcohol, which was directly used for the next step of conversion to the bromide For this purpose, it was heated for  $\frac{1}{2}$  hour on the bath with an equal weight of  $\text{PBr}_3$  The excess reagent was destroyed with ice and the bromide was extracted with ether Distillation at 123-125° and 15 mm yielded 6 gm of  $\beta$ (*p*-ethylphenyl)ethyl bromide

$\text{C}_{15}\text{H}_{15}\text{Br}$  Calculated, C 56.34, H 6.15, found, C 56.65, H 6.20

*1-Methyl-6-ethylphenanthrene*—The Grignard reagent prepared from 2.7 gm of  $\beta$ (*p*-ethylphenyl)ethyl bromide and 0.3 gm of magnesium in 5 cc of ether was chilled to 0° and treated dropwise with 1.2 gm of 2,6-dimethylcyclohexanone (4) in 5 cc of ether After standing overnight at room temperature, the mixture was decomposed with dilute  $\text{H}_2\text{SO}_4$  and extracted with ether The extract was dried and after removal of solvent yielded 3.5 gm of crude carbinol The latter as such was added dropwise with vigorous stirring to 9 cc of 85 per cent  $\text{H}_2\text{SO}_4$  at 0° After 1 hour at room temperature the mixture was extracted with petroleum ether The extract in turn on concentration gave 1.6 gm of crude octahydromethylethylphenanthrene The crude hydrocarbon as such was heated with 1.3 gm of selenium for 6 hours at 300° and then for 1 hour at 340° The reaction mixture was extracted with petroleum ether and the extract was passed through a 2 cm  $\times$  10 cm column of  $\text{Al}_2\text{O}_3$  A fluorescent band which developed was eluted with 20 per cent benzene in petroleum ether 1.2 gm of an oil were obtained after removal of the solvent When this was treated with an equal weight of trinitrobenzene in ethanol, crystallization occurred After two recrystallizations from ethanol, 1.1 gm of the 1-methyl-6-ethylphenanthrene-trinitrobenzene compound were obtained as fine yellow needles, m p 148-149°

The trinitrobenzene complex of the  $\text{C}_{17}\text{H}_{16}$  hydrocarbon from atisine was indistinguishable from this substance and also melted at 148-149° The mixture of the two showed no depression

$\text{C}_{17}\text{H}_{16} \cdot \text{C}_6\text{H}_3\text{O}_6\text{N}_3$  Calculated, C 63.71, H 4.42, found, C 63.57, H 4.24

A portion of the synthetic trinitrobenzene complex was decomposed by passage of its benzene solution through an  $\text{Al}_2\text{O}_3$  column The hydrocarbon which first emerged after removal of solvent was distilled at 0.2

mm and 120°. This material was converted to the picrate in alcoholic solution. After recrystallization from ethanol it formed orange needles at melting point 134–136°. The picrate of the  $C_{17}H_{16}$  hydrocarbon from atisine also melted at 134–136° and a mixture of the two melted at the same point.

$C_{17}H_{16}$   $C_6H_3O_7N_3$  Calculated, C 61.44, H 4.26, found, C 61.61, H 4.15

The hydrocarbon which was regenerated from the picrate with  $Al_2O_3$  was obtained first as an oil which crystallized from ethanol as pearly plates at melting point 43–44°. The melting point of a mixture with the atisine hydrocarbon (m.p. 41–43°) was 41–44°.

$C_{17}H_{16}$  Calculated, C 92.68, H 7.32, found, C 92.74, H 7.25

*$\beta$ (p-Tolyl)ethyl Bromide*—To a well stirred solution of the Grignard reagent from 50 gm of p-bromotoluene and 7 gm of magnesium in 200 cc of ether 13 gm of ethylene oxide in 100 cc of ether were added dropwise. During the process the temperature was kept below 0°. After standing overnight, the mixture was refluxed for 1 hour, 200 cc of benzene were then added, and the mixture was distilled until the vapors reached 65°. Cold dilute  $H_2SO_4$  was added and the oily layer was separated. The dried oil was distilled at 15 mm and the fraction (23 gm) at 113–116° was collected. The boiling point of  $\beta$ (p-tolyl)ethanol prepared by Grignard (9) by another method was reported as 115–116° at 13 mm. The bromide was then prepared from the alcohol with  $PBr_3$  as described above for  $\beta$ (p-ethylphenyl)ethyl bromide. The resulting bromide boiled at 105–108° at 15 mm. Shoesmith and Conner (10) reported a boiling point of 103.5–105° at 11 mm.

*Diphenyl-2,3,2',5'-tetracarboxylic Acid*—1,6-Dimethylphenanthrene was prepared by a series of steps which paralleled exactly the procedure described above for the synthesis of 1-methyl-6-ethylphenanthrene in which at the start  $\beta$ (p-tolyl)ethyl bromide was substituted for  $\beta$ (p-ethylphenyl)ethyl bromide. 0.5 gm of 1,6-dimethylphenanthrene was finally obtained from 3.5 gm of  $\beta$ (p-tolyl)ethyl bromide and 1.8 gm of 2,6-dimethylcyclohexanone. The substance crystallized from petroleum ether and melted at 85–88°. When mixed with a sample of 1,6-dimethylphenanthrene (kindly sent to us by Professor R. D. Haworth) it showed no depression.

0.5 gm dissolved in 1.5 cc of acetic acid was heated with 1 gm of  $CrO_3$  dissolved in 1 cc of water and 3 cc of acetic acid for 4 hours. On chilling, 180 mg of yellow plates of the quinone, m.p. 205–206°, were obtained. Haworth *et al.* (5) reported a melting point of 200°.

160 mg of the quinone dissolved in 2 cc of pyridine and 5 cc of water

were heated on the steam bath and 1.2 gm of  $\text{KMnO}_4$  were gradually added in the course of 30 minutes. The  $\text{MnO}_2$  was collected with water and the filtrate was again heated and treated gradually with an additional 0.5 gm of  $\text{KMnO}_4$ . Excess reagent was reduced with  $\text{NaHSO}_3$  and the filtrate was concentrated to small volume. Acidification yielded a crystalline crop which was collected and recrystallized from dilute acetone. 50 mg of needles were obtained, which melted at  $340\text{--}345^\circ$ . No depression was noted when it was mixed with the diphenyltetracarboxylic acid obtained from atisine and in other ways appeared indistinguishable from the latter.

$\text{C}_{16}\text{H}_{10}\text{O}_8$  Calculated, C 58.17, H 3.05, found, C 58.00, H 3.18

The *tetramethyl ester* was prepared in acetone with diazomethane and on recrystallization formed plates from benzene-petroleum ether which melted at  $150\text{--}151^\circ$ . The mixture with the methyl ester (m.p.  $149\text{--}150^\circ$ ) from atisine melted at  $149\text{--}151^\circ$ .

$\text{C}_{20}\text{H}_{14}\text{O}_8$  Calculated, C 62.15, H 4.66, found, C 62.23, H 4.73

All analyses were performed by Mr. D. Rigakos of this laboratory.

#### SUMMARY

The phenanthrene hydrocarbon,  $\text{C}_{17}\text{H}_{12}$ , obtained by dehydrogenation of atisine has been shown to be identical with 1-methyl-6-ethylphenanthrene which has now been synthesized. The diphenyltetracarboxylic acid originally obtained by oxidation of the atisine hydrocarbon has been shown to be the expected diphenyl-2,3,2',5'-tetracarboxylic acid by comparison with the oxidation product of synthetic 1,6-dimethylphenanthrene.

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## THE ACONITE ALKALOIDS

### XIX FURTHER STUDIES WITH DELPHININE DERIVATIVES

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(Received for publication, June 2, 1947)

In repeated attempts to dehydrogenate both aconitine and delphinine with selenium, the product which could be isolated in either case was in an amount so small and of such a character as to make a study unprofitable and of doubtful value. On the other hand, staphisine (1), atisine (2), hetisine (3), and napelline (4) have all given dehydrogenation products which could be further studied. It is possible that the difference in behavior of the two groups of alkaloids, if not due to a divergence in the skeletal structures, is caused by the interference of special features such as the character, number, and positions of the substituents in the more highly substituted aconitine and delphinine. It appeared therefore worth the attempt to simplify the molecule in the latter by the preliminary removal of some of these groups. With this in view, a further study of the degradation of delphinine has been made through its derivative,  $\alpha$ -oxodelphinine.

Although the formation of  $\alpha$ -oxodelphinine from the alkaloid has introduced the additional oxo group, this was compensated for by the removal of acetic acid on pyrolysis to pyro- $\alpha$ -oxodelphinine. As discussed below, this loss of acetic acid is accompanied by the production of a double bond. The previously described (5) isomerization of the pyro derivative with HCl in methanol has since been found to proceed very quickly and the resulting substance is now called *isopyrooxodelphinine*. The latter must be an intermediate in the formation of the previously reported chloro derivatives (6) obtained by the action of HCl in methanol on pyro- $\alpha$ -oxodelphinine. In particular, the substance  $C_{29}H_{33}O_6NCl_2$  appeared of interest for further degradation attempts. In this substance, two of the four methoxyl groups have been replaced by Cl. Following preliminary studies, it was found possible to remove the halogen with zinc dust in acetic acid. The exact nature of the resulting crystalline substance was somewhat difficult to interpret because of the different ways in which the Cl could be substituted. Most probable appeared the replacement of 1 Cl atom by H and another Cl atom by acetoxy, due to interchange with dissolved zinc acetate. Titration experiments showed definitely the consumption of 2 equivalents of alkali as required by the benzoyl and a new acetoxy group. Analytical results appeared to favor this inter-

pretation and the formulation  $C_{31}H_{37}O_8N$ . In this substance there should still be present the original double bond of pyro- $\alpha$ -oxodelphinine, as well as those of the benzoyl group. A second possibility which has been considered is the removal of HCl with the formation of a new double bond and a formulation  $C_{31}H_{35}O_8N$ . The difficulty at this point of purification, and of making certain of homogeneity, prevented a final decision with the substance itself.

Hydrogenation resulted in a crystalline substance which analyzed well for  $C_{31}H_{45}O_8N$ . In some of the hydrogenation experiments, the apparent hydrogen absorption approximated 5 moles which, in addition to the 3 moles of  $H_2$  required by the benzoyl group, would indicate the absorption of 2 more moles and support the above doubly unsaturated formulation  $C_{31}H_{35}O_8N$ . But of greater importance was the correct interpretation of the formulation of the hydrogenation product as  $C_{31}H_{45}O_8N$ , which would result by the complete hydrogenation of either of the above precursors. This substance will be given the trivial name *hexahydrobenzoyloxodelphinine acetate*. On treatment with alkali the hexahydrobenzoyl and acetyl groups were readily removed with the formation of a crystalline *oxodelphinine*,  $C_{22}H_{33}O_6N$  (m p 306–312°). Aside from stereochemical or other possible rearrangements, this substance, in addition to the original oxo, hydroxyl, and remaining two methoxyl groups of pyrooxodelphinine, has hydroxyl groups in place of a methoxyl group and the benzoyloxy group, and H atoms in place of a methoxyl group and the original acetoxy group.

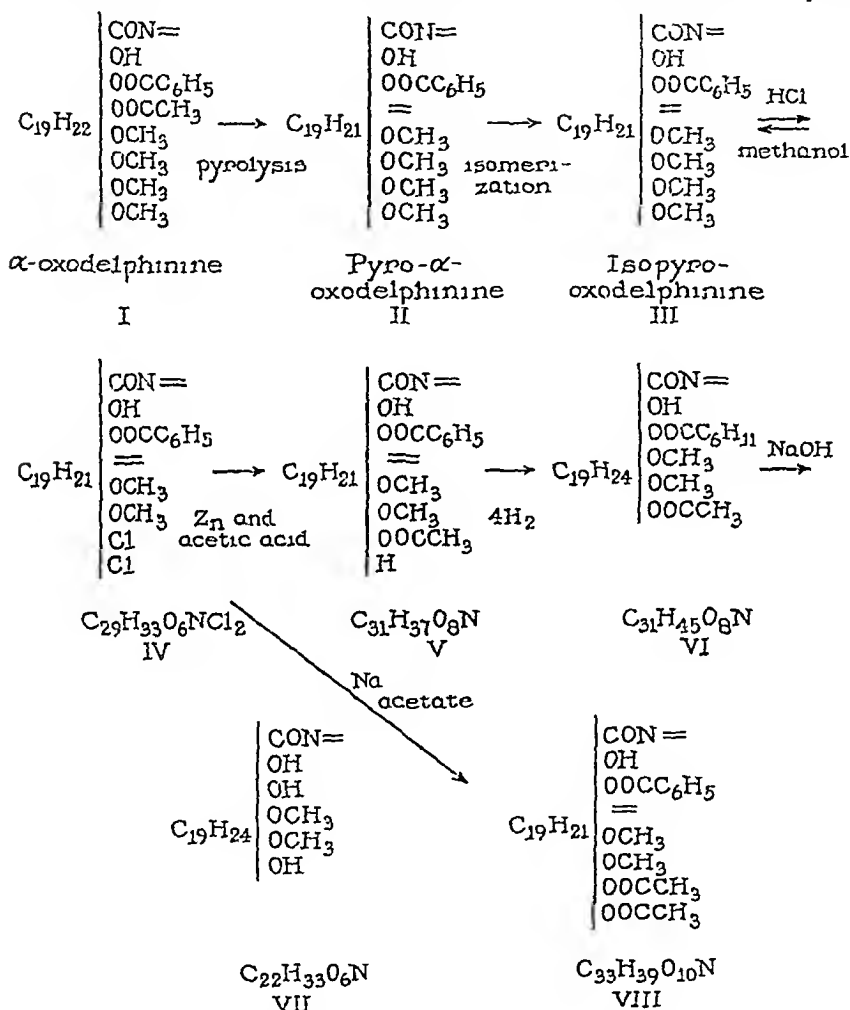
These transformations starting from  $\alpha$ -oxodelphinine are indicated schematically as given in Formulas I to VII. In the case of the substance in Formula V, the formulation  $C_{31}H_{37}O_8N$  is assumed with only 1 double bond.

It was previously (6) shown that the dichloro derivative on heating with methanol could be reconverted to its precursor now called isopyrooxodelphinine by the replacement of the Cl atoms by methoxyl groups. The reactivity of the groups in the positions subject to these transformations has been shown further by the ready conversion of the substance  $C_{29}H_{33}O_6NCl_2$  with sodium acetate in acetic acid to the *diacetoxy derivative*  $C_{33}H_{39}O_{10}N$  (Formula VIII).

At this point advantage was taken of the opportunity to test the effect, if any, of the partial removal of the groups from delphinine on the course of the selenium dehydrogenation. For this purpose, the unsaponified hexahydrobenzoyloxodelphinine acetate,  $C_{31}H_{45}O_8N$ , was used.

A reaction mixture was obtained which was definitely larger in amount and more workable than in the case of the original alkaloid. In several attempts it was possible to isolate by distillation and chromatography a

neutral hydrocarbon fraction which appeared characteristic and possibly homogeneous. Analysis and the molecular weight determination indicated a hydrocarbon,  $C_{17}H_{24}$ . This was supported by the analysis of its picrate



The ultraviolet absorption spectrum of this material is shown in Fig 1 is strongly suggestive of a benzenoid structure and the formulation  $C_{17}H_{21}$  would indicate the presence of two other rings or much less probably double bonds. Since this substance is extremely resistant to further dehydrogenation, at least in the temperature range employed, the two non-aromatic rings may be 5-membered. With the amount of material available, it has not been possible to go beyond the present point of general orientation. Whether the hydrocarbon is a direct fragment of the original molecule

or a somewhat unrelated artifact must await further study. It is in a different category from the phenanthrene hydrocarbons obtained from the other group of aconite alkaloids. However, its production from the delphinine derivative represents some progress over the experience with the alkaloid itself. As opportunity presents, the attempt will therefore be made to extend the study to other derivatives in which additional groups have been removed from the alkaloid.

The isolation of the above oxodelphonine,  $C_{22}H_{33}O_6N$ , is the first experience with a delphinine saponification product which could be ob-

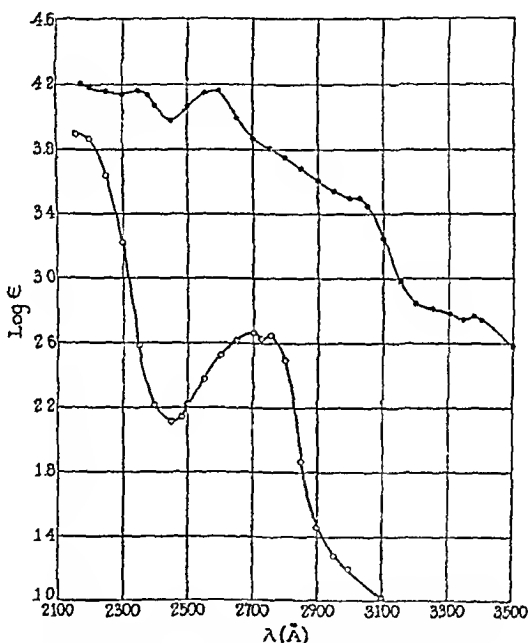


FIG 1 ○,  $C_{17}H_{14}$  hydrocarbon, ●, fluorescent hydrocarbon from the dehydrogenation (calculated for an assumed molecular weight of 220)

tained crystalline. Thus the previously recorded delphonine, pyrodelphonine, and  $\alpha$ -oxodelphonine were obtained only as resins (7). In a more recent attempt with pyro- $\alpha$ -oxodelphonine its saponification product *pyro- $\alpha$ -oxodelphonine* likewise could not be crystallized. On the other hand, consistent with the above experience several other derivatives of the *iso* series have now been obtained crystalline. Isopyrooxodelphonine on saponification has yielded a crystalline *isopyrooxodelphonine*,  $C_{21}H_{35}O_7N$  (m p  $219^\circ$ ).

In connection with the correct interpretation of the degree of unsaturation

of the above substance  $C_{31}H_{37}O_8N$  (or  $C_{31}H_{35}O_8N$ ) it was necessary to return to a study of pyro- $\alpha$ -oxodelphinine, since the former was obtained from the latter through the intermediate dichloro derivative,  $C_{29}H_{33}O_6NCl_2$ . Our recent work has shown that the loss of acetic acid on pyrolysis of  $\alpha$ -oxodelphinine to yield pyro- $\alpha$ -oxodelphinine is accompanied by the production of a double bond. The previously described so called hexahydropyro- $\alpha$ -oxodelphinine (5) has been reinterpreted as an *octahydropyro- $\alpha$ -oxodelphinine*,  $C_{31}H_{47}O_8N$ . Similarly when the isomer isopyro-oxodelphinine is hydrogenated, 4 moles of  $H_2$  are absorbed with the production of *octahydroisopyrooxodelphinine* (m p 212–216°). The latter on saponification to remove hexahydrobenzoic acid yields crystalline *dihydroisopyrooxodelphinine* (m p 197–198.5°).

Although an opportunity has not been found to recheck the presence of double bonds in the pyro derivatives of the alkaloids themselves such as pyrodelphinine or pyroaconitine, there appears no longer reason to assume that their formation proceeds differently from what has been shown in the case of the above oxidation products.

At this point experience in a somewhat unrelated category with certain delphinine derivatives will be recorded. In earlier work (5) the oxidation of delphinine with permanganate was shown to yield the isomeric neutral derivatives  $\alpha$ - and  $\beta$ -oxodelphinine.  $\alpha$ -Oxodelphinine, although still a  $C_{33}$  derivative, no longer analyzed for an N-methyl derivative as in the case of delphinine itself. This paralleled the experience with oxonitine and oxoaconitine, the oxidation products of aconitine, as well as in other recorded instances. It was assumed that  $\beta$ -oxodelphinine behaved likewise. However, this has now been found to be incorrect. The occasion was taken recently to complete the analytical study of a series of delphinine derivatives to aid in the interpretation of their interrelationships.  $\beta$ -Oxodelphinine as well as the methylbenzoyl- $\beta$ -oxodelphinine obtained from it by replacement of acetoxyl by a methoxyl group has now been analyzed as N-methyl derivatives. The results of these analyses are presented in Table I.

The previously recorded  $\gamma$ -oxodelphinine and its derivative, methylbenzoyl- $\gamma$ -oxodelphinine, have also analyzed as N-methyl compounds. In the earlier account of these substances, a close similarity in properties, except in rotation, to  $\beta$ -oxodelphinine and its derivative was reported and this is again shown by the N-methyl determinations. It appears very probable that the two series differ only in a configurational way. On the other hand, there must be a more pronounced structural divergence from  $\alpha$ -oxodelphinine and from oxonitine and oxoaconitine. It appears that in the latter cases their formation involves a rearrangement of the N-alkyl group or that this alkyl group functions as a cross link between the N and

the rest of the molecule and is the point of oxidation. It is also possible that the so called N-alkyl group is really a somewhat labile C-alkyl group which is stabilized by the oxidation.

## EXPERIMENTAL

*Octahydriopyro- $\alpha$ -oxodelphinine*—A repetition of the previously (5) reported hydrogenation with platinum oxide confirmed the absorption of 4 moles

TABLE I  
*N-Methyl and Methoxyl Analyses*

	Found		Calculated	
	NCH <sub>3</sub>	OCH <sub>3</sub>	NCH <sub>3</sub>	OCH <sub>3</sub>
Delphinine	2 36 2 30 2 54	20 40 20 38 20 87	2 51	20 71
$\alpha$ -Oxodelphinine	0 52	20 90	2 45	20 23
Methylbenzoyl $\alpha$ -oxodelphinine	0 69	26 17	2 56	26 47
$\beta$ -Oxodelphinine	2 04 2 04 2 00	20 40 19 77 20 00	2 45	20 23
Methylbenzoyl $\beta$ -oxodelphinine	2 25	26 75	2 56	26 47
$\gamma$ -Oxodelphinine	2 17	19 81	2 45	20 23
Methylbenzoyl- $\gamma$ -oxodelphinine	2 44	25 86	2 56	26 47
Oxomitine, C <sub>33</sub> H <sub>43</sub> O <sub>1</sub> N	0 35*	19 60	2 32*	19 22
Oxoaconitine, C <sub>34</sub> H <sub>45</sub> O <sub>1</sub> N	0 63*	19 17	2 28*	18 82
	0 61	19 50		

\* The N-alkyl determinations with oxomitine and oxoaconitine are given here as N-CH<sub>3</sub> since the procedure for N C H<sub>5</sub> was not used in these cases. The calculated N-alkyl is also given as N-CH<sub>3</sub>, although if present it should be N C H<sub>5</sub>.

of H<sub>2</sub> in excess of the catalyst requirements, *i e.*, 0.2 gm. of pyro- $\alpha$ -oxodelphinine absorbed 40 cc. at 22°, m.p. 186–189° after preliminary sintering.

C <sub>31</sub> H <sub>47</sub> O <sub>8</sub> N	Calculated	C 66.26, H 8.44
	Found	" 66.48, " 8.43
		" 66.50, " 8.46

*Isopyrooxodelphinine*—This substance is formed more readily than at first realized from pyro- $\alpha$ -oxodelphinine. On warming in 30 parts of 3 per cent HCl in methanol, the latter dissolves but is soon replaced by a very sparingly soluble powder of wedge-shaped crystals. This material is slightly contaminated with the previously described sparingly soluble

chloro derivative,  $C_{30}H_{36}O_7NCl$  (6), which is difficult to remove by recrystallization

The melting point<sup>1</sup> was found to be 292–296°

$[\alpha]_D^{25} = -13^\circ$ ( $c = 0.98$ in pyridine)	
$C_{31}H_{39}O_8N$	Calculated C 67.23, H 7.10
	Found " 66.82, " 7.07

For comparison, the rotation of pyro- $\alpha$ -oxodelphinine was determined

$$[\alpha]_D^{25} = +71^\circ (c = 1.04 \text{ in pyridine})$$

*Isopyrooxodelphinine*—0.1 gm of isopyrooxodelphinine was refluxed in a mixture of 7 cc of methanol and 0.5 cc of 10 per cent NaOH for 20 minutes, during which it gradually dissolved. The diluted mixture was extracted with chloroform. The extract on concentration left a resin which, when redissolved in acetone and allowed to evaporate, yielded a crystalline mass. The crystals were somewhat soluble in acetone and were more readily manipulated from an acetone-ether mixture from which it formed needles which were solvent-free. It melted at 219° after slight preliminary sintering.

$C_{41}H_{53}O_7N$	Calculated C 64.10, H 7.85
	Found " 64.37, " 8.08
	" 63.70, " 7.90

*Octahydroisopyrooxodelphinine*—The hydrogenation of 0.2 gm of isopyrooxodelphinine in acetic acid with 50 mg of platinum oxide catalyst was completed after about an hour. The absorption was somewhat in excess of 4 moles. After filtering from catalyst and concentration *in vacuo* the residue readily crystallized under ether. It formed solvent-free triangular micro platelets from chloroform-ether which melted at 212–216° after preliminary softening, especially above 209°.

$C_{31}H_{47}O_8N$	Calculated C 66.26, H 8.44
	Found " 66.23, " 8.44

*Dihydroisopyrooxodelphinine*—70 mg of the previous octahydro derivative readily dissolved in a mixture of 2 cc of methanol and 0.5 cc of 10 per cent NaOH. On bringing to a boil, some odor of hexahydrobenzoic acid was at once apparent. After short boiling, cleavage was complete. The mixture was somewhat diluted and the methanol was boiled off. On being cooled the mixture remained clear. After acidification with HCl the strongly smelling hexahydrobenzoic acid was first removed with petroleum ether. The aqueous phase was then extracted with chloroform.

<sup>1</sup> All melting points reported, unless otherwise stated, are corrected micro melting points.



The latter yielded a residue which crystallized from an acetone-ether mixture. On recrystallization it formed solvent-free micro prisms and platelets which melted at 197–198.5° after slight preliminary sintering.

$C_{24}H_{37}O_7N$	Calculated	C 63.81, H 8.26
	Found	" 63.48, " 8.26

*Diacetate Derivative,  $C_{33}H_{39}O_{10}N$* —The previously described substance,  $C_{29}H_{33}O_6NCl_2$  (6), was treated as follows. 0.1 gm. was refluxed in 5 cc. of acetic acid with 0.5 gm. of fused sodium acetate for 3 hours, during which NaCl separated. The diluted mixture was extracted with chloroform, which in turn was washed free of acetic acid. The extract after concentration to small volume and addition of ether crystallized as micro rods or short flat prisms. It did not melt sharply. After preliminary sintering above 235°, it softened, especially above 270°, and sank to a resin at 275–280°.

$C_{33}H_{39}O_{10}N$	Calculated	C 64.99, H 6.45
	Found	" 64.95, " 6.59

11.84 mg. of substance were refluxed 2.5 hours in a mixture of 0.7 cc. of alcohol and 1.200 cc. of 0.1 N NaOH and titrated back against phenolphthalein. Alkali consumed 0.617 cc., calculated for 3 equivalents 0.584 cc.

*Substance  $C_{31}H_{37}O_8N$  (?)*—0.5 gm. of the dichloro derivative  $C_{29}H_{33}O_6NCl_2$  were refluxed with 2.6 gm. of zinc dust in 25 cc. of acetic acid for 3 hours. After separation from unchanged zinc the diluted mixture was extracted with chloroform. The latter was washed in turn with water, dilute  $Na_2CO_3$ , and water, and concentrated. The substance crystallized on careful addition of ether. It separated from chloroform-ether as a crust or powder of compact microcrystalline aggregates in which individual crystals were difficult to discern. The melting point in a capillary was not sharp and after preliminary discoloration and sintering, especially above 230°, the substance gradually shrank to a resin which slowly melted at 243–248° to a liquid filled with bubbles (uncorrected). Under the microscope after some preliminary softening above 240° and especially above 245°, it gradually melted at 250–260° but crystals remained until 266° (corrected). Recrystallization did not appear to alter the behavior. The substance was halogen-free.

For analysis it was dried at 110° and 0.2 mm.

$C_{31}H_{37}O_8N$	Calculated	C 67.48, H 6.76, $OCH_3$ 11.25
$C_{31}H_{35}O_8N$	"	" 67.72, " 6.42, " 11.30
	Found	(a) " 67.35, " 6.30, " 10.52
		(b) " 67.51, " 6.60
		(c) " 66.80, " 6.60

? 165 mg. of substance (a) were refluxed for 1 hour in 2 cc. of ethanol and

2.07 cc of 0.1 N NaOH and titrated back against phenolphthalein. Calculated for 2 equivalents, 0.44 cc, found 0.46 cc.

*Hexahydrobenzoyloxodedelphonine Acetate*,  $C_{31}H_{45}O_8N$ —The hydrogenation of the previous  $C_{31}H_{37}O_8N$  derivative proceeded readily in acetic acid. 0.1 gm with 50 mg of platinum oxide absorbed within 1 hour 19 cc of  $H_2$  in excess of the catalyst requirement. Calculated for 4 moles of  $H_2$ , about 17 cc. The filtrate from the catalyst on concentration left a residue which crystallized under ether. On recrystallization by addition of ether to the concentrated solution in acetone it separated as minute micro, mostly triangular, platelets, which melted at  $187^\circ$  after some preliminary softening above  $183^\circ$ .

In another experiment with 3.94 gm of substance, the recorded absorption of  $H_2$  with 0.2 gm of catalyst was about 813 cc of  $H_2$  for the substance. The approximate 4 mole requirement is 658 cc, and for 5 moles 822 cc. In other experiments the observed absorption again approached the 5 mole requirement.

$C_{31}H_{45}O_8N$	Calculated	C 66.50, H 8.11
	Found (a)	" 66.39, " 8.07
	(b)	" 66.48, " 8.07

*Oxodedelphonine*,  $C_{22}H_{33}O_6N$ —0.72 gm of the preceding substance was dissolved in 15 cc of methanol and treated while warm with 2 cc of 10 per cent NaOH. After refluxing for 15 minutes the mixture was nearly neutralized with  $H_2SO_4$  and concentrated to remove methanol. The mixture, which smelled strongly of hexahydrobenzoic acid, deposited a crop of small glistening rods and prisms. 0.24 gm was collected with water. The mother liquor on concentration yielded a second crop. For recrystallization it was dissolved in hot somewhat diluted methanol. After further dilution and boiling off the methanol, the substance crystallized from about 5 cc of  $H_2O$  as small flat prisms, or 4-sided often rhombic platelets which were anhydrous and melted at  $306\text{--}312^\circ$  after some preliminary sintering above  $292^\circ$ .

$C_{22}H_{33}O_6N$	Calculated	C 64.82, H 8.17
	Found (a)	" 64.87, " 8.28
	(b)	" 64.88, " 8.30

*Dehydrogenation of Hexahydrobenzoyloxodedelphonine Acetate*—1.63 gm of the substance  $C_{31}H_{45}O_8N$  were ground with 2.9 gm of selenium and heated in a sealed tube at  $330^\circ$  for 6 hours. The residue was pulverized and then exhaustively extracted with hot benzene. The extract was shaken with 10 per cent HCl. The aqueous phase was concentrated to a few cc, made alkaline, and then extracted with ether. The latter yielded only 5 mg of a yellow resin from which nothing crystalline was obtained. The

benzene phase was then shaken with 5 per cent NaOH which, in turn, was acidified and repeatedly extracted with ether. On evaporation, the latter yielded 60 mg of a brown liquid in which the odor of hexahydrobenzoic acid was apparent. This fraction when dissolved in the minimum of hot  $\text{H}_2\text{O}$  yielded 15 mg of benzoic acid, m p 122–124°.

The remaining benzene solution of neutral material on evaporation left 250 mg of a brown oil. It was redissolved in benzene and passed through a 12 mm  $\times$  10 cm column of  $\text{Al}_2\text{O}_3$  and then developed with benzene under ultraviolet light. The first blue fluorescing zone eluted with benzene yielded 125 mg of a yellow oil. A second fraction of 15 mg of a yellow resin then followed. A dark colored zone which remained at the top of the column was eluted with 2 per cent methanol in benzene and gave 100 mg of a black tar. But these last two fractions proved to be too unpromising for further study.

The first fraction was then rechromatographed from petroleum ether solution on  $\text{Al}_2\text{O}_3$  and developed under ultraviolet light, at first with petroleum ether which yielded 70 mg of a non-fluorescing oil. The blue fluorescing zone was then eluted with 10 per cent benzene in petroleum ether and gave 40 mg of material. The non-fluorescing fraction was treated with an equal weight of picric acid in ethanol and the yellow crystals obtained were recrystallized from ethanol. This material appeared to be a loosely bound derivative. When crushed between the glass slides for the micro melting point determination, on the hot stage, the impression was obtained that it had already become a mixture of yellow needles of picric acid and an oil. The apparent melting point was 121°.

$\text{C}_7\text{H}_8$ , $\text{C}_6\text{H}_5\text{O}\cdot\text{N}_3$	Calculated	C 60.35, H 5.97
	Found	" 60.31, " 5.63

The hydrocarbon was regenerated from the picrate by passing its benzene solution through  $\text{Al}_2\text{O}_3$ . The resulting colorless oil (35 mg) was distilled in a sublimation apparatus at 90° and 0.02 mm.

$\text{C}_7\text{H}_{12}$	Calculated	C 89.40, H 10.60
	Found	" 89.48, " 10.50

A second dehydrogenation experiment with 2 gm of starting material and 4 gm of selenium was conducted for a longer time at 325° for 16 hours and then at 340° for 3 hours. The reaction mixture was worked up as previously. By chromatographing the neutral fraction on  $\text{Al}_2\text{O}_3$ , 100 mg of the non-fluorescing hydrocarbon were obtained by elution with petroleum ether and 15 mg of the blue fluorescing fraction by elution with 10 per cent benzene in petroleum ether. Addition of an equal weight of trinitrobenzene to the latter in ethanol yielded 5 mg of yellow needles, m p 140–148°. Recrystallization from ethanol gave 3 mg of yellow needles, m p 155–163°.

The substance was probably not homogeneous and the small amount made further purification impossible. The analytical data are nevertheless recorded with a possible interpretation

$C_{18}H_{18}$	$C_6H_3O_6N_3$	Calculated	C 64.40, H 4.74
		Found	" 64.26, " 4.73

80 mg of the non-fluorescing hydrocarbon were again heated with 160 mg of selenium in a sealed tube at  $325^\circ$  for 20 hours and at  $340^\circ$  for 6 hours. The benzene extract of the reaction mixture on concentration yielded a residue which was dissolved in petroleum ether and passed through a 12 mm  $\times$  10 cm column of  $Al_2O_3$ . 65 mg of a colorless non-fluorescing oil were eluted with the petroleum ether and were followed by 7 mg of a blue fluorescing substance when 10 per cent benzene in petroleum ether was used. This last material gave no crystalline derivatives and was unquestionably a complex mixture. Its absorption spectrum (Fig 1) was not suggestive. The non-fluorescing fraction was distilled at  $90^\circ$  and 0.02 mm.

$C_{17}H_{14}$	Calculated	C 89.40, H 10.60
	Found	" 89.50, " 10.60

The molecular weight was determined in camphor. Calculated, 228.2, found, 235. The absorption spectrum of this substance is recorded in Fig 1.

All of the analyses have been performed by Mr D Rigakos of this laboratory.

#### SUMMARY

The failure of previous attempts to dehydrogenate aconitine and delphinine with selenium was caused possibly in part by the number and positions occupied by the substituting groups. Initial efforts have been made to achieve simpler derivatives for further degradation studies by systematic removal of such substituents. In the present work pyro- $\alpha$ -oxodelphinine, now shown to be an unsaturated pyrolysis product of  $\alpha$ -oxodelphinine, has been used as the starting point. After isomerization with acid to isopyrooxodelphinine, a series of transformations was made which yielded finally a substance  $C_{22}H_{33}O_6N$  in which some of the original O-containing groups have been removed. Dehydrogenation of an ester of the latter has yielded a hydrocarbon,  $C_{17}H_{14}$ , the absorption spectrum of which suggests that it may be a bicyclopentobenzene and in a different category from the phenanthrene hydrocarbons obtained from the atisine group of aconite bases.

The saponification products of a number of the isopyrrolodelphinine series have been obtained crystalline

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# THE ANTIPODAL SPECIFICITY AND INHIBITION OF CRYSTALLINE CARBOXYPEPTIDASE

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(Received for publication, May 5, 1947)

Investigations of the enzymatic specificities of crystalline proteolytic enzymes toward synthetic peptide derivatives have shown that, as a rule, catalysis is limited to those substrates which contain the enzyme-specific amino acid residue in the L configuration, the D isomers of these substrates being entirely resistant to hydrolysis (1). This antipodal specificity was explained by Bergmann and coworkers (2-4) by the polyaffinity theory, according to which enzyme-substrate combination requires a fixed asymmetric arrangement of the active groups of the substrate and a complementary arrangement of the active groups of the enzyme, such that the binding planes described by the respective active groups can approach each other to within a few Ångström units. In the case of the D antipodes, steric hindrance caused by the protrusion of the relatively large enzyme-specific amino acid residue is supposed to interfere with a close approach between the enzyme and the reactive groups of the substrate.

More recently, Bergmann and Fruton (5) reported inhibition of the enzymatic hydrolysis of benzoyl-L-tyrosylglycylamide by the corresponding D antipode, no hydrolysis occurring when either the racemate or equimolar mixtures of the L and D isomers of this peptide were tested with chymotrypsin or papain. Stahmann, Fruton, and Bergmann (6) reported that the addition of an equimolar concentration of carbobenzoxyglycyl-D-phenylalanine to the L isomer decreased by about 60 per cent the first order reaction constant of the hydrolysis of the latter by carboxypeptidase.<sup>1</sup>

Both phenomena were tentatively explained by the assumption of the formation in solution of a stable racemate, the affinities of the optical antipodes for each other being greater than that of the L antipode for the enzyme (5, 6). The validity of this hypothesis for the antipodal inhibition of carboxypeptidase may be questioned on the grounds that carbobenzoxyglycylphenylalanine is an anion and that electrostatic repulsion between the isomers would counteract any tendency for association.<sup>2</sup> Stahmann,

<sup>1</sup> Similar inhibition observed on the addition of relatively hydrolysis-resistant carbobenzoxyglycyl amino acids to the L phenylalanine homologue was ascribed by these authors to a different mechanism.

<sup>2</sup> Similar considerations have been previously applied by Jacobson (7) to the positively charged benzoyl-DL arginamide which, unlike the uncharged benzoyl-DL-tyrosylglycylamide (5), is readily hydrolyzed by trypsin.

Fruton, and Bergmann (6) offer the alternative suggestion that the inactive D isomer of this substrate combines with the enzyme at but one point, whereas Pauling (8) proposes that in cases in which antipodal inhibition has been observed the enzyme is structurally complementary to a strained configuration of the L isomer, intermediate in configuration between those of the L and D forms

A systematic investigation of the mechanism of inhibition of carboxypeptidase, now under way in this laboratory, has led to a reinvestigation of the reported antipodal inhibition of the hydrolysis of carbobenzoxyglycylphenylalanine (CGlyP). The data that have been obtained are at variance with those previously reported (6) and appeared of sufficient importance for the problem of antipodal inhibition to be presented herein in some detail

#### EXPERIMENTAL

**Substrates**—The synthesis of carbobenzoxyglycylphenylalanine from carbobenzoxyglycyl chloride and phenylalanine ethyl ester was carried out essentially as described by Hofmann and Bergmann (9). However, in view of the brevity of their account some essential details are given herein. Following the addition of pyridine to carbobenzoxyglycylphenylalanine ethyl ester, prepared from 1.9 gm. of phenylalanine ethyl ester hydrochloride, the ether layer was removed and washed consecutively with water, dilute HCl, water, sodium bicarbonate, and water, and then dried over anhydrous sodium sulfate. Upon removal of the ether *in vacuo*, the peptide ester prepared from racemic phenylalanine crystallized, whereas those prepared from the optical antipodes of phenylalanine remained as an oil. After one recrystallization from ethanol-water, the melting point of carbobenzoxyglycyl-DL-phenylalanine ethyl ester was 90–91° (corrected) and the nitrogen content 7.2 per cent (calculated 7.3 per cent).

Saponification of the peptide ester was effected by dissolving it in 50 cc. of absolute ethanol, followed by the addition of 15 cc. of 1 N NaOH. The mixture was shaken and then acidified with HCl to Congo red, as described previously (9), and concentrated *in vacuo* until crystallization began. Carbobenzoxyglycylphenylalanine was recrystallized from a mixture of ethyl acetate and ethyl ether. Maximum final yield, 2.8 gm. from 1.9 gm. of phenylalanine ethyl ester hydrochloride (95 per cent of theoretical yield).

This procedure was followed for the synthesis of the dipeptide from L-, D-, and DL-phenylalanine<sup>3</sup>. Both isomers and the racemate had identical nitrogen contents of 7.8 per cent (calculated 7.8 per cent). The corrected melting points of the L and D isomers were identical, *i.e.* 125–126°, whereas

<sup>3</sup> L-Phenylalanine was prepared in this laboratory by Mr. John E. Snoko by resolution of the racemate. D-Phenylalanine was obtained through the courtesy of Dr. W. H. Stein of The Rockefeller Institute for Medical Research.

that of the racemate was 159.5–160.5°. Mixtures of the racemate and the L isomer melted over a wide range (125–150°). The optical rotations of the L and D isomers in absolute ethanol were, respectively,  $[\alpha]_D^{25} = +36^\circ$  and  $-39^\circ$ , as compared to  $+38.5^\circ$  reported by Hofmann and Bergmann (9) for the L isomer.

**Enzyme**—Crystalline carboxypeptidase was prepared from frozen beef pancreas glands as previously described (10) except for the following modifications. Recrystallization was effected by alternating the procedures of (a) extraction of the crystals with 0.2 N LiOH up to pH 10, followed by crystallization on the addition of acetic acid to pH 7.6 to 8.0, and (b) dissolving the crystals in 10 per cent LiCl, followed by graded dialysis in the cold against 5 per cent LiCl, 2 per cent NaCl, and distilled water. In either case, dissolution of the crystals occurred more rapidly and completely in the cold (ice-water mixture) than at room temperature. Six recrystallizations were sufficient to increase the proteolytic coefficient of the enzyme toward CGlyP to a maximum level.

**Methods**—Enzymatic measurements were carried out at 25° in a 0.033 M phosphate buffer, pH 7.5. Substrate concentration was about 0.05 M with respect to the L isomer. The progress of hydrolysis was measured on 0.2 cc aliquots with the manometric ninhydrin method (11). Enzyme solutions were prepared daily from a stock solution containing about 0.3 to 0.5 mg of N per cc. The latter was prepared about every 3rd day from a stock suspension of crystals. Enzyme nitrogen concentrations were determined with the semimicro-Kjeldahl method.

### Results

In agreement with previous data (9, 10, 12) it was found that over a wide range<sup>4</sup> the hydrolysis of L-CGlyP by crystalline carboxypeptidase follows the kinetics of a first order reaction. The mean reaction constant,  $k$ , was determined for each experiment from the slope of the straight lines obtained when  $\log_{10} (100/(100 - \text{per cent hydrolysis}))$  was plotted against  $t$ , the time in minutes. First order reaction kinetics<sup>5</sup> were likewise observed for the enzymatic hydrolysis of racemic CGlyP as well as for mixtures of the L and D isomers of this substrate. Representative data are plotted in Fig. 1.

A summary of all kinetic experiments is given in Table I. Proteolytic coefficients,  $C$ , were calculated in the conventional manner (12), in which the first order reaction constant per mg of enzyme nitrogen per cc is rep-

<sup>4</sup> Departure from linearity was observed when hydrolysis exceeded about 80 per cent (cf Fig. 1).

<sup>5</sup> In all experiments in which the D isomer was present, rates of hydrolysis were calculated with respect to the concentration of the L isomer.



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resented by  $k/(mg \text{ of enzyme } N \text{ per cc})$ . The mean value for the proteolytic coefficient of carboxypeptidase toward L-CGlyP, with three different enzyme concentrations, is comparable to that previously reported (10), *i.e.* 11.5. While the enzyme is entirely inactive toward the D antipode of this substrate, it is as active toward the racemic mixture as toward solutions containing only the L isomer. The mean proteolytic coefficient<sup>5</sup> obtained from measurements with two different enzyme preparations, and in different concentrations of one of these, is 12.3. Approximately the same value was obtained when the activity of carboxypeptidase was measured in solutions containing varying absolute and relative concentrations of the L and D isomers (Table I).

#### DISCUSSION

The results obtained in this investigation are at variance with those previously reported (6) and fail to provide any evidence for the antipodal inhibition of the activity of carboxypeptidase by the D isomer of CGlyP. Within the limits of the experimental error, the proteolytic coefficients for the hydrolysis of L-, DL-, and mixtures of L- and D-CGlyP, are the same, independent of enzyme concentration and independent also of the relative concentrations of the two isomers in the reaction mixture. In the light of these findings, the system carboxypeptidase-CGlyP need no longer be considered an exception to Bergmann's theory of antipodal specificity of enzyme action. It also follows from the present results that the racemate of CGlyP is as suitable a substrate for kinetic studies on carboxypeptidase as the L isomer. The use of the racemate as a test substrate is of practical advantage, since the available methods for the resolution of racemic phenylalanine are considerably laborious and mostly inefficient.

It is beyond the scope of the present investigation to explain the divergent results reported by Stahmann and coworkers (6). Confidence in the present data may be derived from the use of the more specific and accurate ninhydrin method, as compared to the alcohol titration method of Grassmann and Heyde (13), for measuring the progress of hydrolysis of the substrate. The enzymatic purity of six times recrystallized carboxypeptidase used in this work was the same as that previously obtained on the eight times recrystallized enzyme (10), probably because of the more efficient recrystallization procedure which has been employed herein. While in the light of previous experience (10) the enzymatic purity of a three times recrystallized preparation, as used by Stahmann and coworkers (6), may be questioned, the evidence is insufficient to ascribe their results to this factor.

This work has been supported by grants from the Rockefeller Foundation,

the Division of Grants, National Institute of Health, United States Public Health Service, and from the Duke University Research Council

#### SUMMARY

Kinetic studies on the hydrolysis of L-, D-, DL-, and mixtures of L- and D-carbobenzoxylglycylphenylalanine by six times recrystallized carboxypeptidase are reported. In contradistinction to previous reports, it was found that the rate of hydrolysis of the L isomer is independent of the presence in the reaction mixture of the D isomer of this substrate, although, in agreement with previous data, the enzyme was found to be entirely inactive toward the D isomer. Since no evidence for antipodal inhibition was found, the system carboxypeptidase-carbobenzoxylglycylphenylalanine need no longer be considered an exception to Bergmann's theory of antipodal specificity of enzyme action. Hence the racemic mixture of this substrate appears a valid testing material for enzymatic studies. The physical properties of carbobenzoxylglycyl-DL-phenylalanine ethyl ester and of carbobenzoxylglycyl-DL-phenylalanine have been described.

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# STUDIES ON PROTEOLYTIC ACTIVITY OF CRYSTALLINE PROTEIN B PREPARED FROM BEEF PANCREAS\*

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(Received for publication, June 2, 1947)

Last year a new crystalline protein from beef pancreas was isolated in this laboratory (1). Freshly prepared protein showed high thymonucleodepolymerase activity which decreased sharply during subsequent recrystallizations.

It was originally thought that the crystalline protein represented a partially inactivated thymonucleodepolymerase. Later, it was shown (2) that, with more precautions during recrystallization, the nucleolytic activity accumulated in the mother liquor, it was concluded, therefore, that the crystalline protein is not a nuclease.<sup>1</sup> It was also noticed that the crystalline protein exhibited proteolytic activity, and this activity (3) could be considerably increased by treatment either with crystalline trypsin or with an aqueous extract of duodenal mucosa.

For reasons of simplicity, the new crystalline protein will be referred to in this paper as protein B, or zymogen B. The object of this paper is to present a more detailed study of the proteolytic action of zymogen B and of its activated form.

## Methods

In the early experiments, zymogen B was prepared according to the method previously described (1). Later, the method of preparation was modified as follows:

The precipitate, obtained in the third step of McCarty's method (4), was dissolved in 4 volumes of water plus 1 volume of 0.1 acetate buffer, pH 4, the solution was adjusted to pH 4. For each 100 cc. of solution, 25 cc. of a saturated solution of ammonium sulfite were added. The precipitate (Fraction A) was centrifuged off and washed twice, each time with one-half of the previous volume of 20 per cent saturated ammonium sulfate, containing 20 per cent of 0.1 acetate buffer, pH 4.

\* Aided by grants from the Donner Foundation, Inc. and the John and Mary R. Markle Foundation.

† Some of the data included in this report were taken from a thesis submitted by Cecilia K. Keith to the Graduate School of Marquette University in partial fulfillment of the degree of Master of Science.

<sup>1</sup> Since that time, nuclease has been considerably purified (unpublished).

The filtrate and washings were combined, pH was adjusted to 6.5 with 5 N sodium hydroxide, and a saturated solution of ammonium sulfate was added to make the solution 40 per cent saturated with respect to ammonium sulfate, a correction was made for the volume of sodium hydroxide added. The precipitate was filtered and washed with one-half of the original volume of 40 per cent saturated ammonium sulfate, containing 20 per cent of M acetate buffer, pH 6.5. The combined liquid and washings (Fraction C) were used for the further purification of the nuclease.

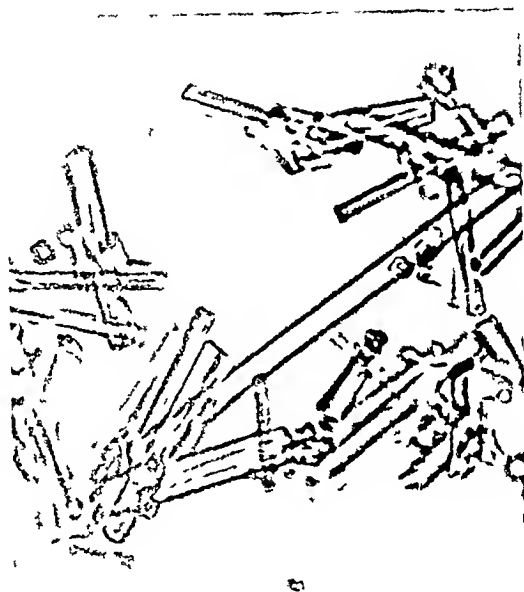


FIG. 1. Protein B (form  $B_1$ ), three times recrystallized,  $\times 1000$ .

The washed precipitate (Fraction B) consisted almost entirely of protein B, which was obtained in two different crystalline forms, referred to as  $B_1$  (small needles, Fig. 1) and  $B_2$  (plates, photograph published previously (1)).

The washed precipitate B was dissolved in a minimal amount of water, the water being added first a few cc. at a time, then drop by drop. When a clear solution was obtained, the pH was adjusted to 5.5 with N sulfuric acid, this resulted in the formation of a crystalline precipitate  $B_1$ . The tube was left at room temperature for 24 hours to complete the crystallization and was then centrifuged. The mother liquor was dialyzed against 0.01 M acetate buffer, pH 5.5, for 5 days in the cold. A partially crystalline precipitate resulted which was recrystallized as previously described (1), and yielded typical plates of the  $B_2$  form.

Since protein  $B_1$  was obtained in a different crystalline form and under

somewhat different conditions than protein B<sub>2</sub> (ammonium sulfate concentration about 25 per cent of saturation for the former and none for the latter), it was originally suspected that the two proteins may not be identical. Their behavior toward activation and inhibition was, however, very similar. The final proof of identity was obtained in the following manner.

Protein B<sub>1</sub> was recrystallized three times by dissolving the crystals in 25 per cent saturated ammonium sulfate at pH 6.5, and then bringing the pH down to 5.5. The final crystalline product, which was of the B<sub>1</sub> form, was dissolved in water and acetic acid at pH 4, and dialyzed against 0.01 M

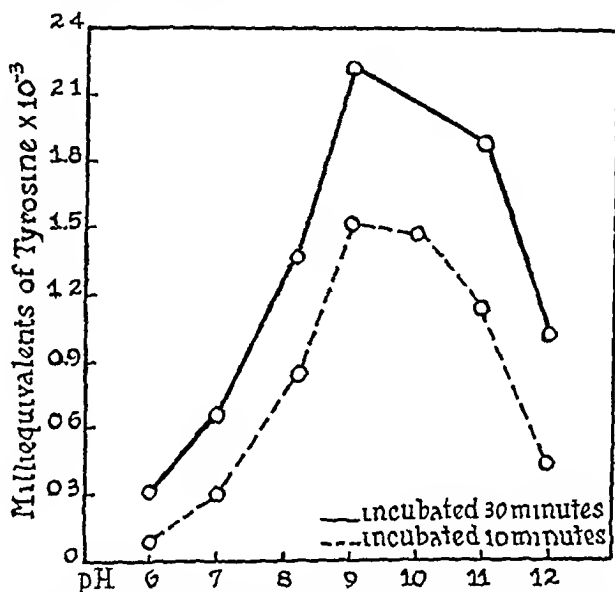


FIG. 2. Influence of pH on activity. All tubes contained 2.5 cc of 0.5 per cent casein, 0.5 cc of enzyme solution, and 2.0 cc of appropriate buffer. pH was measured by means of a glass electrode.

acetate buffer, pH 5.5. This resulted in a crystalline precipitate which upon microscopic examination, proved to be typical plates of the B<sub>2</sub> form. These crystals were recrystallized twice by dissolving in dilute acetic acid at pH 4 and bringing the pH up to 5.5. The final crystals of the B<sub>2</sub> form were dissolved in 25 per cent saturated ammonium sulfate at pH 6.5, the pH was brought down to 5.5. Typical needles of the B<sub>1</sub> form were obtained. In the description of the experimental findings, the symbols B<sub>1</sub> and B<sub>2</sub> were retained to indicate a difference in the method of preparation.

The proteolytic activity was determined as follows: 2.5 cc of 0.5 per cent casein solution, adjusted to pH 9, were placed in a 15 cc centrifuge tube and mixed with 2 cc of 0.2 M borate buffer, pH 9, and 0.5 cc of enzyme

solution, diluted according to the expected proteolytic activity. The mixture was incubated 10 minutes at 37°, following which it was treated with 5 cc of a 20 per cent trichloroacetic acid. The undigested casein was centrifuged, 2 cc of supernatant liquid were transferred to a Klett tube

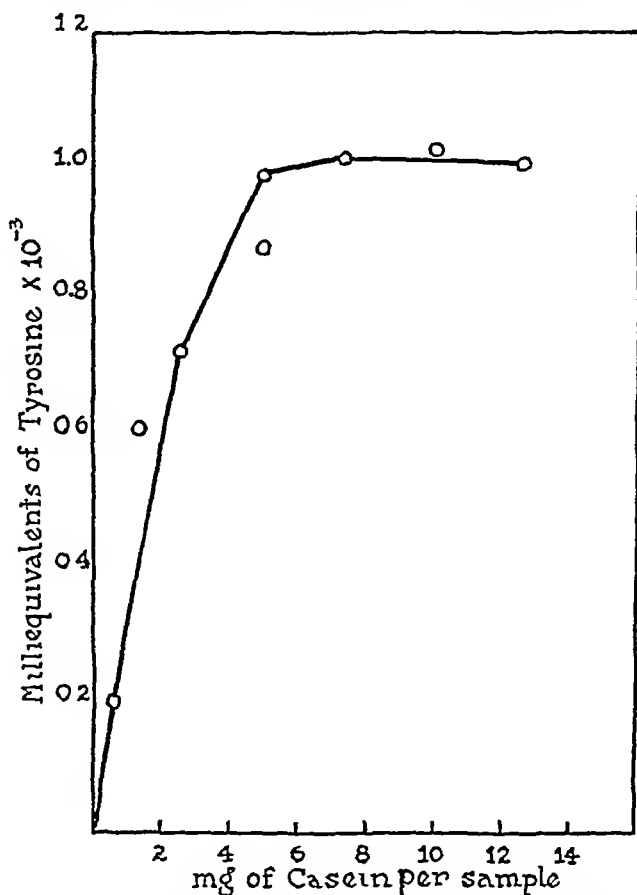


FIG 3 Influence of the concentration of substrate on activity. All tubes contained 0.5 cc of a solution of zymogen B<sub>2</sub>, Sample 8, 2.5 cc of casein solution of appropriate strength, and 2.0 cc of 0.2 N borate buffer, pH 9. The enzyme solution contained 0.1 mg of protein per cc.

and treated with 4 cc of N sodium hydroxide and 1 cc of the phenol reagent of Folin and Ciocalteu (5), diluted with an equal volume of water. The color was read in a Klett photoelectric colorimeter and was compared with a standard curve for tyrosine. A control tube, containing the same quantities of buffer and casein solutions, was set up for each experimental tube. The enzyme was added to the control tube after the addition of trichloro-

acetic acid The tyrosine value found in the control tube was subtracted from the value found for the experimental tube

The enzymic activity was expressed in tyrosine units 1 tyrosine unit is defined as the amount of enzyme which, under the previously specified conditions, will liberate  $1 \times 10^{-3}$  miliequivalent of acid-soluble tyrosine per sample The choice of such a unit was based on the results of experiments performed on unactivated zymogen B, and shown in Figs 2, 3, and 4

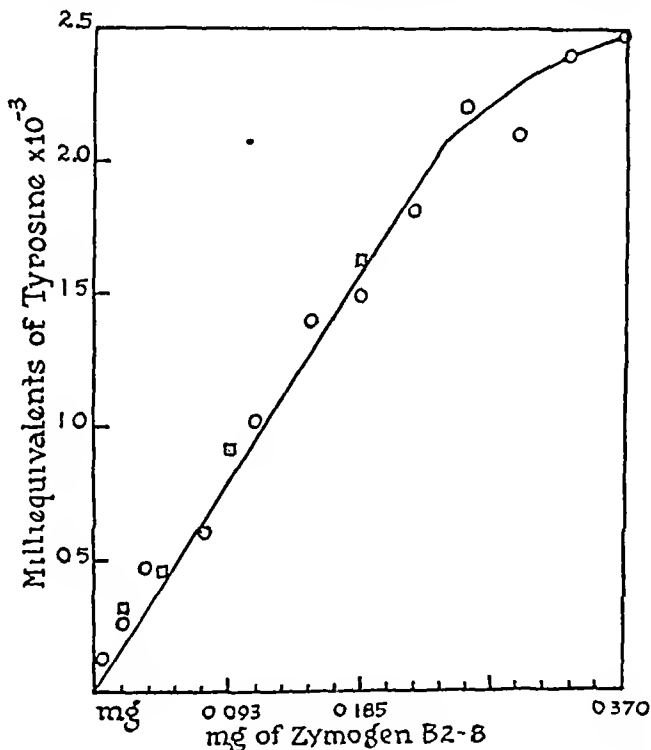


FIG 4 Influence of enzyme concentration on activity All tubes contained 2.5 cc of 0.5 per cent casein solution, 2.0 cc of 0.2 M borate buffer, pH 9, 0.5 cc of a solution of zymogen B<sub>2</sub>, Sample 8, of appropriate strength

A limitation, however, was introduced in view of the experiments shown in Fig 4 The proportionality between the amount of enzyme used and the amount of liberated tyrosine existed only below the value of 2 units per sample In order to simplify the direct comparison of the activity of different samples, only the readings which fell within this limit were used Under the conditions of the experiments, only readings of less than 150 on the Klett scale were considered to be accurate



## EXPERIMENTAL

The activation experiments were carried out with crystalline trypsin, crystalline chymotrypsin, crude aqueous extract of duodenal mucosa, and

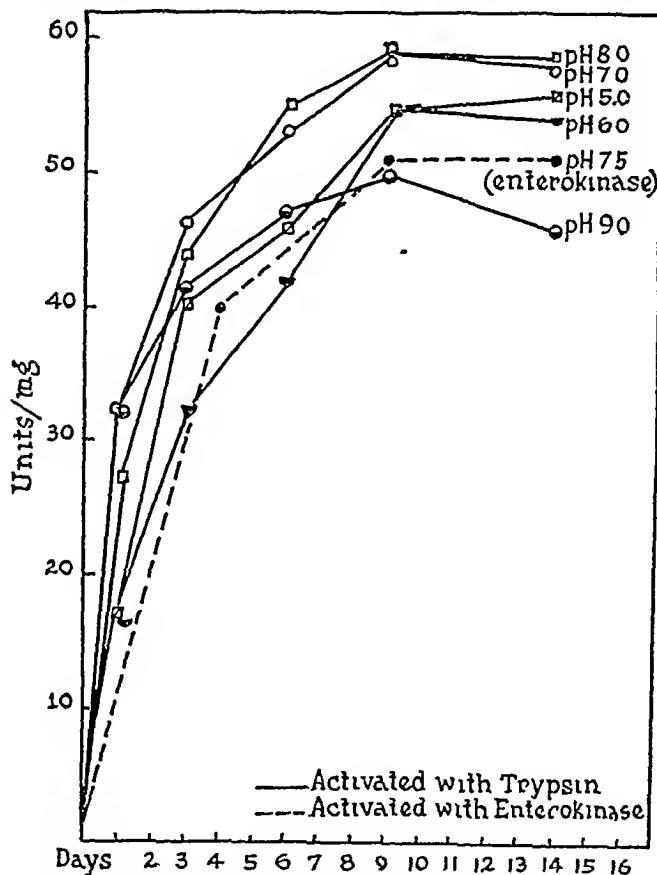


FIG 5 Activation of protein B<sub>2</sub>, Sample 14, with crystalline trypsin, prepared by C K K, and purified enterokinase, obtained from Dr M Kunitz. The solution of zymogen B contained 12.25 mg of protein per cc. 1 cc of this solution was mixed with 1 cc of an appropriate buffer solution: M acetate, M phosphate, 0.2 M borate, and 1 cc of trypsin solution, containing 0.02 mg per cc. For determination of activity, 0.3 cc of this solution was withdrawn, diluted either 1/10 or 1/100, and tested. In the experiment with enterokinase, 1 cc of the solution containing 1 mg was substituted for the trypsin solution.

purified enterokinase<sup>2</sup>. Chymotrypsin was inactive, *i.e.*, it neither increased nor decreased the proteolytic activity already present in zymogen

<sup>2</sup> We are greatly indebted to Dr M Kunitz, who supplied us with samples of crystalline trypsin, crystalline soy bean trypsin inhibitor, and purified enterokinase.

B The amount of trypsin, which produced a strong activation and yet did not produce any detectable proteolytic activity in the control tubes, was approximately 1 part of crystalline trypsin per 500 parts of zymogen. All samples were activated in the refrigerator at temperatures close to 5°, and were tested as previously described.

For each activation experiment, two controls were set up. One contained a solution of zymogen and water instead of trypsin solution, and served as a control for self-activation. In none of these experiments was any substantial self-activation demonstrated. Also, there was no loss in the proteolytic activity already present in the zymogen. The second control contained trypsin and water instead of the solution of zymogen. It was noticed, however, that in these very dilute solutions trypsin deteriorated rapidly. Therefore, the trypsin control was made up fresh for each determination.

The activation was carried out at different pH values (Fig. 5). The maximal value of the proteolytic activity was achieved after 10 days of activation. The maximal proteolytic activity was about 20 to 30 times higher than the activity of the original zymogen. Activation occurred in all pH values studied. Both the final activity and the velocity of activation were similar throughout the entire range investigated. A very similar curve was obtained on activation with enterokinase.

An analogous experiment in which eight times recrystallized chymotrypsinogen, prepared according to the method of Kunitz and Northrop (6), was activated by trypsin, is shown in Fig. 6. The general similarity to the previously discussed experiment is apparent. There are, however, considerable quantitative differences. The final maximal value of proteolytic activity was higher with chymotrypsinogen than with zymogen B. The velocity of activation with chymotrypsinogen was also higher than in the case of zymogen B, particularly at pH 9. No significant activation of chymotrypsinogen occurred at pH 5, and only slight activation occurred after treatment with enterokinase. After comparing the results of these two experiments (Figs. 5 and 6), the conclusion was reached that zymogen B and chymotrypsinogen belong to a similar type of zymogen, but are not identical.

The optimal pH of the activated protein B was pH 8 (Fig. 7, scale 10 times higher than in Fig. 2), instead of pH 9 as for the unactivated zymogen (Fig. 2). It is not possible to say definitely whether this shift in optimal pH was significant, particularly in view of the fact that much larger amounts of zymogen were required for the determination of activity.

The maximal activation of protein B ever achieved by any activating agent accounted for approximately 40 per cent of the activity of crystalline chymotrypsin (Table I) on the basis of the protein nitrogen present in the

solution. This final value for activity was therefore sufficiently high to exclude the possibility of contamination with chymotrypsinogen. Furthermore, the activity was fairly constant in five different preparations of zymogen B. Thus far, however, we have been unable to crystallize active

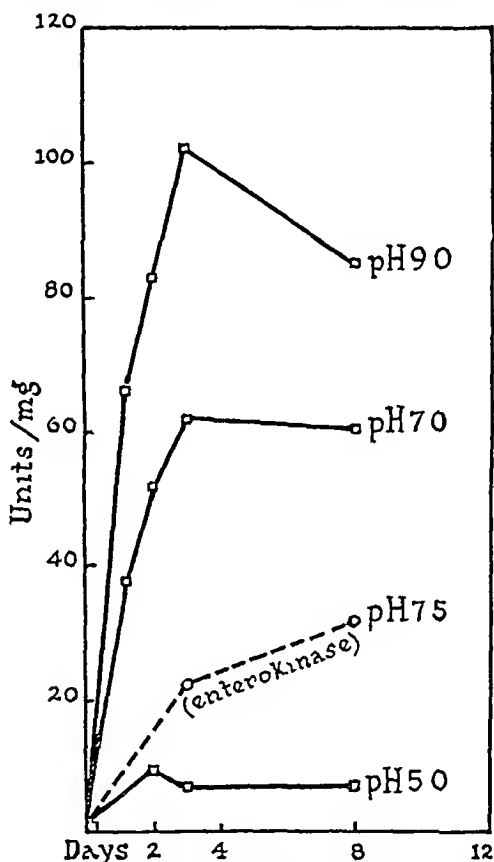


FIG 6 Activation of eight times recrystallized chymotrypsinogen with crystalline trypsin and purified enterokinase. The solution of chymotrypsinogen contained 7 mg per cc. All other conditions were similar to those for Fig 5.

enzyme B. Consequently, the figures recorded in Table I should be considered as having only relative value.

In order to characterize protein B still further, the influence of crystalline soy bean trypsin inhibitor on the activated enzyme B was studied. Fig 8 shows the results of such an experiment. The data are expressed in per cent of non-inhibited units. It is obvious that the trypsin inhibitor does not form a stable compound with enzyme B as it does with trypsin (7). It is also obvious that the trypsin inhibitor reacts in a similar manner with

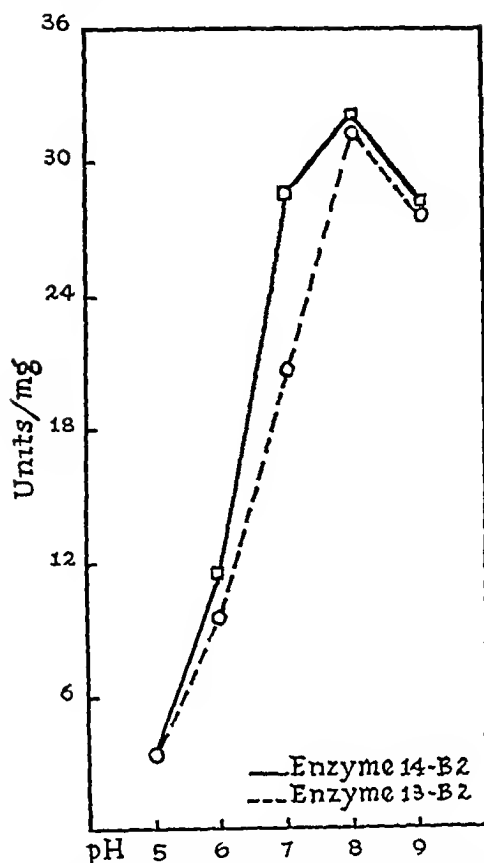


FIG 7 Effect of pH on activated zymogen B. Each tube contained 0.06 mg. of activated protein B in an appropriate buffer solution, 2.5 cc. of 0.5 per cent casein solution. Total volume was 5 cc. Incubation period was 10 minutes at 37°.

TABLE I

Comparison of Maximal Final Activity of Activated Protein B with Activity of Crystalline Chymotrypsin

Enzyme	Tyrosine units per mg	Activity in per cent of activity of chymotrypsin
Chymotrypsin	134	100
Protein B <sub>1</sub> , Sample 15, recrystallized 3 times	47	36
" B <sub>2</sub> , " 8, " 2 "	44	33
" " " 13, " 2 "	30	22
" " " 14, " 3 "	59	44
" " " 15, " 4 "	46	34

enzyme B and with chymotrypsin, forming with both a dissociable complex. The affinity of trypsin inhibitor for enzyme B was, however, greater than

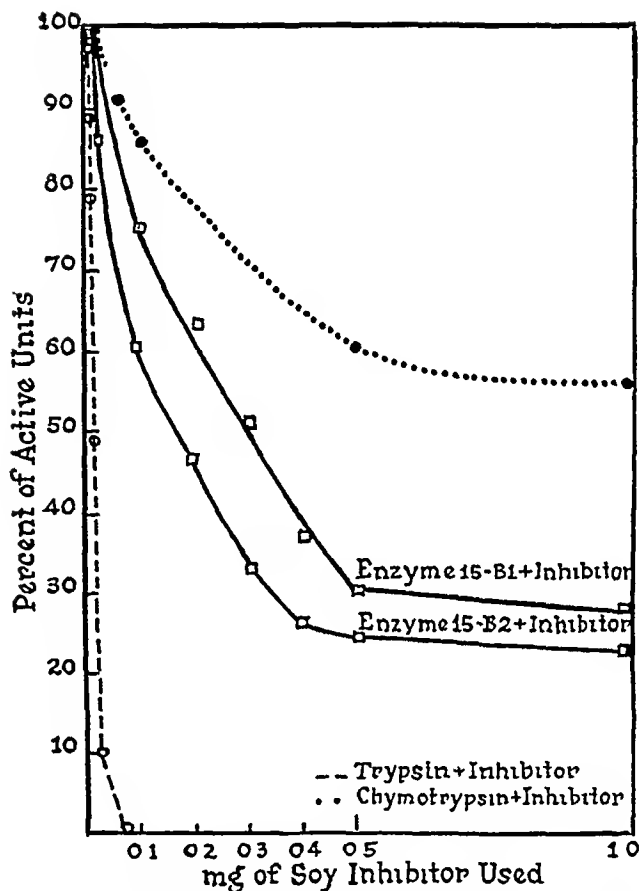


FIG 8 The effect of crystalline soy bean trypsin inhibitor on crystalline trypsin, protein B, and crystalline chymotrypsin. The solution of crystalline chymotrypsin contained 0.009 mg per cc. 0.1 cc of this solution was mixed with 2.5 cc of 0.5 per cent casein solution, 2.0 cc of borate buffer, various amounts of the diluted solution of trypsin inhibitor, and enough water to make 5 cc. Incubation period was 10 minutes at 37°. The solution of protein B (form B<sub>2</sub>) contained 0.039 mg per cc, the solution of protein B<sub>1</sub> contained 0.036 mg of protein per cc. All other conditions for the experiment were the same as for the experiment with chymotrypsin. Three different trypsin preparations were used. Two of these were prepared by us and the third was obtained from Dr. Kunitz. This last proved to be the most active. The solutions of trypsin were therefore adjusted to contain 1.5 trypsin units per cc. On this basis, all three preparations gave identical results with trypsin inhibitor.

for chymotrypsin. The results of the experiments on inhibition also indicate that activated enzyme B and chymotrypsin are similar, but not the same substance.

## DISCUSSION

In view of the evidence which has been presented, protein B has been identified as a proteolytic zymogen. No definite opinion, however, can be expressed concerning the inclusion of protein B in a group of chymotrypsins. Until the specificity of this enzyme for some well defined linkage in the peptide chain is known, this problem will remain open. Therefore, it seems advisable not to introduce a definite name for protein B until that time.

The second point to be discussed is concerned with the nucleolytic activity of protein B. The discussion is limited to the preparations obtained in the B<sub>2</sub> form, no attempts have been made to determine the nucleolytic activity of the B<sub>1</sub> form of crystals. In a previous paper of this series (1) the assumption has been made that protein B represents a partially inactivated thymonucleodepolymerase. Upon this assumption, many efforts were made to find, and if possible to restore, the damaged functional groups. All attempts to do so have failed.

In view of the recent findings, several of the past difficulties can be explained. The proteolytic activity of a dialyzed solution of protein B was slight at pH 4. It increased considerably at pH 5.5, at which pH recrystallization was usually carried out. When recrystallization was carried out at a lower temperature and at as low a pH as possible, the recovery of the nucleolytic activity in the mother liquor was considerably improved (2).

In a series of experiments, the loss of nucleolytic activity of the crystalline protein B was tested. The solution of protein B was exposed to room temperature for 2 hours at different pH values. Fairly good correlation with the curve representing the proteolytic activity was found. The destruction of nucleolytic activity was not much higher in the tubes which contained activating amounts of trypsin, indicating that the destruction was due to the proteolytic action of protein B itself. The addition of crude duodenal extract slowed down the rate of destruction, most probably because it supplied a competitive substrate.

As yet, it is not possible to make a definite statement regarding the amount of nuclease contaminating protein B. It does not seem likely, however, that the contamination could have been greater than 1 per cent.

## SUMMARY

The new crystalline protein obtained from beef pancreas, provisionally called protein B, has been identified as a proteolytic zymogen.

Protein B has been activated by crystalline trypsin and by purified enterokinase. The highest activity observed after complete activation was 59 tyrosine units per mg. of protein, and accounted for 44 per cent of the activity of crystalline chymotrypsin.

Comparison of the behavior during activation, as well as behavior toward crystalline soy bean trypsin inhibitor, led to the conclusion that protein B is not identical with chymotrypsinogen. The activated form of this protein is also not identical with chymotrypsin.

The previous finding of nucleolytic activity associated with protein B (the B<sub>2</sub> form) as an impurity was discussed.

The authors wish to thank M<sup>r</sup> L. C. Massopust for the photograph and drawings.

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# A DIETARY FACTOR ESSENTIAL FOR GUINEA PIGS

## XII THE *IN VITRO* INFLUENCE OF THE ANTISTIFFNESS FACTOR UPON THE METABOLISM OF LIVER TISSUE

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(Received for publication, June 11, 1947)

The *in vitro* action of fat-soluble vitamins upon the glycolytic processes of liver tissues obtained from animals deficient in these essential metabolites is difficult to determine, due to the fact that these substances are not readily soluble in the usual substrates. Houchin (1) reported the effect of  $\alpha$ -tocopherol upon the oxygen consumption of dystrophic muscle when a suspension of this compound is added to muscle slices. Only inconclusive results were obtained. The addition of  $\alpha$ -tocopherol disodium phosphate to homogenized muscle tissue reduced the increased succinoxidase activity of vitamin E-deficient muscle.

Van Wagtendonk and Simonsen (2) have found that a deficiency of the antistiffness factor results in a derangement of the respiratory and glycolysis rates in the liver. It was postulated that the antistiffness factor plays an important rôle in the energy metabolism through its influence upon the adenylic acid system, and consequently would regulate the anaerobic and aerobic breakdown of carbohydrates. If, therefore, the antistiffness factor would be added *in vitro* to the metabolizing tissue preparations, it might exert a direct influence upon these processes. The solution of the fat-soluble compounds tested was achieved by grinding these into the tissues while preparing the homogenate, thus dissolving them in the tissue fat. A direct effect upon the anaerobic glycolysis could be established. The use of the homogenate technique in a study of this type has been shown by Potter (3-5) to be a valid means for comparison of different respiratory and glycolytic functions. While intact cell structure is necessary for some types of biochemical activity, several reactions involving the oxidative synthesis of high energy phosphate bonds have been demonstrated in homogenates. Liver preparations have been shown to maintain their ability to catalyze the synthesis of adenosine triphosphate in the absence of intact cell structure (4).

### EXPERIMENTAL

The syndrome typical of the antistiffness factor deficiency was developed in the test animals by feeding them for a period of 6 to 20 weeks on the diet

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described by van Wagendonk (6) Stock animals were fed a diet consisting of rolled barley and fresh green vegetables *ad libitum* Animals, weighing 400 to 600 gm each, were killed by a blow on the head, and the liver was rapidly removed Approximately  $1.20 \pm 0.005$  gm of tissue were weighed on a chainomatic balance and transferred to a cold mincing tube (3)<sup>1</sup> containing 2.00 cc of Krebs and Henseleit Ringer's solution (8) The tissue was minced for exactly 3 minutes, and diluted to such a volume with Ringer's solution that 1 cc contained 100 mg of tissue The suspension was thoroughly mixed 2 cc of this mince and 1 cc of Ringer's solution were added to each of the Warburg flasks The flasks were attached to the manometers, placed in the water bath, and the system was charged with a gas mixture composed of 95 per cent nitrogen and 5 per cent carbon dioxide saturated with water vapor at 37° The mixture had previously been purified by passing it through a purifying unit, as described by Savage and Ordal (9) Six flasks were run with each determination and measurements of carbon dioxide output were recorded at 5 minute intervals Glycolysis rates of normal and deficient animals were compared and the effect of adding the antistiffness factor and related materials directly to the homogenates of deficient and normal liver samples was determined

The addition of the compounds to be tested was accomplished as follows To a dry mincing tube, 0.02 cc of a petroleum ether solution, containing 0.10 mg per cc of the solution to be tested, was added from a micro pipette The solvent was evaporated, leaving the material deposited on the bottom of the tube The last traces of the solvent were removed *in vacuo* (0.1 mm) The tissue was placed in the tube and the grinding operation was carried out as described above<sup>2</sup> For purposes of comparison a control tube was run without the added factor The liver tissue for this control was taken from the same liver lobe as that used in the test

The substances tested were as follows a natural fraction (m p 82.5-84°) identified as a C<sub>28</sub> or C<sub>29</sub> unbranched aliphatic alcohol, probably still containing traces of the high melting active fraction,<sup>3</sup> a synthetic unbranched aliphatic alcohol (C<sub>29</sub>H<sub>59</sub>OH),<sup>4</sup> a natural factor (m p 164°),<sup>3</sup> ergosterol,<sup>5</sup> and cholesterol<sup>5</sup>

<sup>1</sup> It is very important that cold conditions be maintained throughout the whole preparation of the homogenate All of the operations were carried out in a cold box maintained at 0-5° (7) All glassware and reagents used in preparing the homogenates were also chilled before use

<sup>2</sup> The final concentration of added material in each flask is 0.333 γ The flask contains 200 mg of tissue in a total volume of 3 cc

<sup>3</sup> van Wagendonk, W. J., and Ross, L. E., to be published

<sup>4</sup> Prepared by Dr. R. G. Jones, Eli Lilly and Company

<sup>5</sup> Both were commercial preparations (Eastman Kodak Company) and were used as such without further purification

The data obtained were analyzed statistically by the methods of Fisher (10)

### Results

The results obtained are summarized in Tables I to III. In agreement with the results obtained with liver slices (2), the deficient liver mince showed a decreased rate of anaerobic glycolysis in comparison with that of animals maintained on a stock diet, as summarized in Table I. The  $Q_{CO_2}^{N_2}$  for the stock animals was 1.04, whereas that for the deficient animals was 0.44.

The effects of *in vitro* addition of various metabolites to the tissue minces are summarized in Table II. The addition of the low melting (m.p. 82.5–84°) natural fraction, tentatively identified as a  $C_{29}$  straight chain

TABLE I

*Anaerobic Glycolysis of Liver Homogenates of Normal and Deficient Guinea Pigs*

Diet	No. of determinations*	$Q_{CO_2}^{N_2}$ Mean $\pm$ standard error
Stock	14	1.04 $\pm$ 0.08 (0.43 – 1.52)†
Deficient	26	0.44 $\pm$ 0.03 (0.26 – 0.81)

\* Each determination represents the average of six flasks

† The  $Q_{CO_2}^{N_2}$  was calculated on the dry weight basis, with the factor of 3.71 (2)

‡ The values in parentheses indicate the range

alcohol, had no significant effect upon the  $Q_{CO_2}^{N_2}$ . The synthetic  $C_{29}$  alcohol also did not show any *in vitro* activity. With the high melting fraction, however, a marked increase was shown in each of the determinations. The average anaerobic glycolysis rate for deficient liver minces in this series was 0.43. With the addition of the antistiffness factor, a value of 0.59 was obtained. In Table III, the effects of addition of the antistiffness factor upon the liver tissue of each individual animal are shown. In every case an increase was noted, the average increase being 30 per cent. Ergosterol also proved to be active when added to homogenates of deficient livers. Cholesterol, however, was entirely inactive. No activity could be shown for the antistiffness factor when added to the liver minces from animals raised on the stock diet.

### DISCUSSION

The results of the experiments presented in this paper further extend our knowledge of the action of the antistiffness factor in controlling the inter-

TABLE II

*Anaerobic Glycolysis of Normal and Deficient Liver Homogenated with and without in Vitro Addition of Antistiffness Factor and Other Substances*

Diet	No of determinations*	Compound tested	$Q_{CO_2}^N$ (Mean $\pm$ standard error)
Deficient	6		0 43 $\pm$ 0 04 (0 26 - 0 61)†
		Antistiffness factor, 164°	0 59 $\pm$ 0 05 (0 38 - 0 76)
"	5		0 39 $\pm$ 0 03 (0 27 - 0 47)
		Natural C <sub>23</sub>	0 42 $\pm$ 0 02 (0 41 - 0 45)
"	4		0 44 $\pm$ 0 01 (0 33 - 0 55)
		Synthetic C <sub>23</sub>	0 43 $\pm$ 0 01 (0 33 - 0 52)
"	5		0 39 $\pm$ 0 07 (0 26 - 0 46)
		Ergosterol	0 56 $\pm$ 0 06 (0 51 - 0 68)
"	4		0 41 $\pm$ 0 02 (0 37 - 0 45)
		Cholesterol	0 41 $\pm$ 0 01 (0 39 - 0 45)
Stock	6		1 06 $\pm$ 0 17 (0 53 - 1 52)
		Antistiffness factor, 164°	1 04 $\pm$ 0 17 (0 52 - 1 53)

\* Each determination represents the average of six flasks

† The values in parentheses indicate the range

TABLE III

*Comparison of Rates of Anaerobic Glycolysis of Liver Homogenates from Deficient Gunca Pigs, with and without in Vitro Addition of Antistiffness Factor (164° Melting Point)*

The result represent  $Q_{CO_2}^N$

Time on diet	Without added factor	With added factor	Increase	Per cent increase
<i>weeks</i>				
6	0 45	0 51	0 06	13 3
6	0 55	0 76	0 21	38 2
6	0 43	0 58	0 15	34 9
6	0 61	0 75	0 14	23 0
10	0 43	0 56	0 13	30 2
20	0 26	0 38	0 12	46 2
Mean values	0 455	0 59	0 135	31 0

mediate metabolism of carbohydrate in the liver and partially substantiate the hypotheses as to the action of the factor which have been presented in previous publications (2, 11). It was reported (2) that the increase in the respiratory rate and decrease in the anaerobic glycolysis in deficient liver slices are reversible by the *in vivo* administration of the antistiffness factor for a period of 5 days and that *in vitro* addition of adenosine triphosphate to the metabolizing deficient tissue increased the low  $Q_{CO_2}^{N_2}$ . These changes are correlated with the large decrease in the glycogen storage in the liver. Since the addition of adenosine triphosphate to the metabolizing tissue restores the normal anaerobic glycolysis function of the liver, it was suspected that the activity of the factor was primarily directed toward the maintenance of the adenosine triphosphate level in the tissue. This might be accomplished if the factor acted as the prosthetic group of an enzyme controlling the phosphorylation of the adenylic acid to adenosine diphosphate or the further phosphorylation of adenosine diphosphate to adenosine triphosphate. The factor might also act as an inhibitor to the breakdown of adenosine triphosphate in the animal tissue. The present work further corroborates the view that the factor does act as a part of an enzyme system.

The *in vitro* addition of the antistiffness factor in a concentration of  $3 \times 10^{-7} M$  has a definite influence on the glycolysis rate in deficient tissue slices. This concentration is of the same order of magnitude or less than that proposed by Green (12) as one of the criteria for the classification of a substance as an essential metabolite.

Complete restoration of the  $Q_{CO_2}^{N_2}$  to the normal value was not found upon the addition of the factor. This could not be expected in view of the other gross changes which occur during the deficiency, *i.e.* decreased alkaline serum phosphatase activity (13), abnormal nucleotide distribution (11), and a decrease in the albumin globulin ratio (14). None of these changes could be reversed immediately by the *in vivo* administration of the factor, but require time for the adjustments to occur. One of the main impurities of the original preparation (15), the  $C_{29}$  alcohol, was completely inactive when tested in its synthetic form.

That ergosterol should also be active while cholesterol is not is surprising. However, this might be explained by the fact that a commercial preparation was used. Preliminary identification and absorption spectra of the high melting fraction do indicate a steroid structure, possibly closely related to ergosterol. This work will be the subject of a future publication.

\* The final concentration of the active fraction is 0.333  $\gamma$  per cc. Experiments to be published (van Wagtendonk, W. J., and Ross, L. E.) established this fraction to have a molecular weight of  $\pm 400$ . With this as the basis the active concentration, as used here, is at the most  $3 \times 10^{-7} M$ .

## SUMMARY

A technique is described whereby the *in vitro* effect of a fat-soluble compound on the aerobic and anaerobic metabolism of isolated tissues can be determined. The *in vitro* addition of the antistiffness factor causes a marked and significant increase in the  $Q_{CO_2}^{N_2}$  of liver homogenates from animals deficient in the antistiffness factor.

Grateful acknowledgment is made for the support by grants from the Ella Sachs Plotz Foundation and the National Science Fund of the National Academy of Sciences.

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# THE CLEAVAGE OF THE STEREOISOMERS OF CYSTATHIONINE BY LIVER EXTRACT

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(Received for publication, June 20, 1947)

In an earlier communication (1) it was reported that L-cystathionine, S-(L- $\beta$ -amino- $\beta$ -carboxyethyl)-L-homocysteine, was able to support growth in lieu of cystine in the diet of the growing white rat. However, it was found that L-cystathionine was not able to replace methionine under the same dietary conditions in which homocysteine is capable of supporting growth in the absence of dietary methionine. Thus L-cystathionine is apparently cleaved *in vivo* to cysteine. The enzymatic cleavage of L-cystathionine to L-cysteine by liver tissue coincides with this conclusion (2, 3).

In a later communication (4) the synthesis of the other isomers<sup>1</sup> of cystathionine was described, and growth experiments in which these isomers were fed to young white rats on sulfur-deficient diets were reported. L-Allocystathionine was found to substitute for cystine on the cystine-free, methionine-restricted diet. However, in contrast to the behavior of L-cystathionine, L-allocystathionine did support growth on the methionine-free diet which contained an adequate supply of choline. These facts permitted the inference that L-allocystathionine in the intact animal is cleaved predominantly to homocysteine. On the other hand, neither D-cystathionine nor D-allocystathionine was found to be capable of supporting growth on a cystine-free diet. It was, therefore, thought to be of interest to investigate directly the action of the liver extracts on the isomers of L-cystathionine. The results of this study and their correlation with the previous *in vivo* data are reported in the present communication.

## EXPERIMENTAL

To 20 mg. samples of the isomers of cystathionine in small Erlenmeyer flasks, 20 cc. of M/15 phosphate buffer (pH 7.6) were added. The atmosphere in the flasks was replaced by an oxygen-free mixture. The stoppered flasks were placed in a shaking device and immersed in a water

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<sup>1</sup> D-Cystathionine, S-(D- $\beta$ -amino- $\beta$ -carboxyethyl)-D-homocysteine, D-allocystathionine, S-(D- $\beta$ -amino- $\beta$ -carboxyethyl)-L-homocysteine, L-allocystathionine, S-(L- $\beta$ -amino- $\beta$ -carboxyethyl)-D-homocysteine.

bath maintained at a temperature of 38.4°. After 10 to 15 minutes, a liver extract, prepared according to the directions of Smythe (5), was added in the amounts indicated in Tables I and II. When cyanide was

TABLE I  
*Representative Experiments on Cleavage of D-Cystathionine and L-Allocystathionine*

Substrate	Volume of liver extract per 20 mg substrate	Cyanide	H S test	Sullivan values*	Cleavage	Folin Marenzi values*	Cleavage
	cc			mm $\times 10^{-3}$	per cent	mm $\times 10^{-3}$	per cent
D-Cystathionine	5	—	—	6	13	7	17
	2	—	—	5	11	5	11
	2	+	—	6	13	5	11
L-Allocystathionine	5	—	—	3	7	48	107
	2	—	—	3	7	37	82
	2	+	—	5	11	18	40
None	5	—	—	0		1	

\* Sullivan and Folin-Marenzi values are expressed in millimoles of disulfide to facilitate comparison on an absolute basis of the isomers which yield cysteine and those which yield homocysteine. The 20 mg of substrate are equivalent to 90 mm of cystathionine or 45 mm of disulfide.

TABLE II  
*Representative Experiments on Cleavage of D-Allocystathionine and L-Cystathionine*

Substrate	Volume of liver extract per 20 mg substrate	Cyanide	H <sub>2</sub> S test	Sullivan values*	Cleavage	Folin Marenzi values*	Cleavage
	cc			mm $\times 10^{-3}$	per cent	mm $\times 10^{-3}$	per cent
D-Allocystathionine	5	—	—	17	38	19	42
	2	—	—	12	27	16	36
	2	+	—	7	16	6	13
L-Cystathionine	5	—	+	13	29	22	49
	2	—	+	17	38	19	42
	2	+	—	22	49	26	58
None	2	—	—	1	2	1	2

\* See Table I

to be used, 1 cc of 0.1 M NaCN was introduced at this point. A rapid stream of the gas mixture was passed into the flasks, which were then re-stoppered and shaken in the bath for 2.5 hours. Following the incubation period, a 20 per cent aqueous solution of trichloroacetic acid was added in sufficient volume to yield a final concentration of 5 per cent with respect to trichloroacetic acid. After the mixture had stood for 15 to 20 minutes,

cystine or methionine, the representative experiment given in Table II shows that it is cleaved by the liver extract. In the six experiments in which 5 cc of extract were employed, the cleavage, judging by Sullivan analyses, ranged from 38 to 51 per cent (average 42 per cent). The extent of the cleavage of this isomer is somewhat less than that of its optical isomer, L-allocystathionine. Using smaller volumes of extract reduces the extent of the cleavage of D-allocystathionine. In five experiments with 2 cc of extract in the absence of cyanide, the cleavage ranged from 27 to 47 per cent and averaged 33 per cent (Sullivan values).

Cyanide was found to inhibit the cleavage of L-allocystathionine. In five such experiments in which 2 cc of extract were used in the presence of cyanide, the cleavage ranged from 13 to 18 per cent (average 16 per cent). Thus, the action of cyanide on the cleavage of D-allocystathionine was similar to its action on the cleavage of L-allocystathionine. In five instances in which both the Sullivan and Fohn-Marenzi analyses were carried out, the ratio of cysteine to total disulfides ranged from 75 to 100 per cent (average 86 per cent). Since cysteine accounts for over 75 per cent of the disulfide present in liver digests of this isomer, D-allocystathionine is apparently cleaved predominantly to D-cysteine.

The *in vitro* cleavage of D-allocystathionine clarifies the results obtained in the feeding experiments. The 3-carbon moiety of D-allocystathionine has the unnatural configuration, and, if D-allocystathionine is cleaved *in vivo* as it is *in vitro*, it would not support growth because apparently D-cysteine is not available for growth, since D-cystine does not support growth (11) and only the L moiety of *meso*-cystine is so available.

The cleavage of L-cystathionine under the same conditions was included in the experiment reported in Table II for the purpose of comparing the two isomers which yield cysteine on cleavage. In six experiments in which L-cystathionine was incubated with 5 cc of liver extract, the range of cleavage (judging by Sullivan analyses) was from 29 to 42 per cent (average 33 per cent). Attention is called to the observation that the cleavage of L-cystathionine appears greater when less liver extract is used. In four experiments in which 2 cc of extract were used, the range of cleavage was from 38 to 51 per cent (average 48 per cent).

The presence of desulfurase in liver extracts did not complicate the results obtained on the cleavage of these isomers of L-cystathionine. Desulfurase attacks L-cysteine, producing hydrogen sulfide. The absence of hydrogen sulfide in digests in which homocysteine was produced is consistent with Smythe's observation that desulfurase attacks homocysteine only slightly (5). Apparently D-cysteine is likewise not appreciably attacked by this enzyme, since no hydrogen sulfide was found in digests of D-cystathionine. However, in the case of L-cystathionine hydrogen sulfide tests were always positive when the digestion was carried out in the



bath maintained at a temperature of 38.4° After 10 to 15 minutes, a liver extract, prepared according to the directions of Smythe (5), was added in the amounts indicated in Tables I and II When cyanide was

TABLE I

*Representative Experiments on Cleavage of D-Cystathionine and L-Allocystathionine*

Substrate	Volume of liver extract per 20 mg substrate	Cyanide	H S test	Sullivan values*	Cleavage	Folin Marenzi values*	Cleavage
	cc			mm $\times 10^{-3}$	per cent	mm $\times 10^{-3}$	per cent
D-Cystathionine	5	—	—	6	13	7	17
	2	—	—	5	11	5	11
	2	+	—	6	13	5	11
L-Allocystathionine	5	—	—	3	7	48	107
	2	—	—	3	7	37	82
	2	+	—	5	11	18	40
None	5	—	—	0		1	

\* Sullivan and Folin-Marenzi values are expressed in millimoles of disulfide to facilitate comparison on an absolute basis of the isomers which yield cysteine and those which yield homocysteine The 20 mg of substrate are equivalent to 90 mm of cystathionine or 45 mm of disulfide

TABLE II

*Representative Experiments on Cleavage of D-Allocystathionine and L-Cystathionine*

Substrate	Volume of liver extract per 20 mg substrate	Cyanide	H <sub>2</sub> S test	Sullivan values*	Cleavage	Folin Marenzi values*	Cleavage
	cc			mm $\times 10^{-3}$	per cent	mm $\times 10^{-3}$	per cent
D-Allocystathionine	5	—	—	17	38	19	42
	2	—	—	12	27	16	36
	2	+	—	7	16	6	13
L-Cystathionine	5	—	+	13	29	22	49
	2	—	+	17	38	19	42
	2	+	—	22	49	26	58
None	2	—	—	1	2	1	2

\* See Table I

to be used, 1 cc of 0.1 M NaCN was introduced at this point A rapid stream of the gas mixture was passed into the flasks, which were then re-stoppered and shaken in the bath for 2.5 hours Following the incubation period, a 20 per cent aqueous solution of trichloroacetic acid was added in sufficient volume to yield a final concentration of 5 per cent with respect to trichloroacetic acid After the mixture had stood for 15 to 20 minutes,

cystine or methionine, the representative experiment given in Table II shows that it is cleaved by the liver extract. In the six experiments in which 5 cc of extract were employed, the cleavage, judging by Sullivan analyses, ranged from 38 to 51 per cent (average 42 per cent). The extent of the cleavage of this isomer is somewhat less than that of its optical isomer, L-allocystathionine. Using smaller volumes of extract reduces the extent of the cleavage of D-allocystathionine. In five experiments with 2 cc of extract in the absence of cyanide, the cleavage ranged from 27 to 47 per cent and averaged 33 per cent (Sullivan values).

Cyanide was found to inhibit the cleavage of L-allocystathionine. In five such experiments in which 2 cc of extract were used in the presence of cyanide, the cleavage ranged from 13 to 18 per cent (average 16 per cent). Thus, the action of cyanide on the cleavage of D-allocystathionine was similar to its action on the cleavage of L-allocystathionine. In five instances in which both the Sullivan and Folm-Marenzi analyses were carried out, the ratio of cysteine to total disulfides ranged from 75 to 100 per cent (average 86 per cent). Since cysteine accounts for over 75 per cent of the disulfide present in liver digests of this isomer, D-allocystathionine is apparently cleaved predominantly to D-cysteine.

The *in vitro* cleavage of D-allocystathionine clarifies the results obtained in the feeding experiments. The 3-carbon moiety of D-allocystathionine has the unnatural configuration, and, if D-allocystathionine is cleaved *in vivo* as it is *in vitro*, it would not support growth because apparently D-cysteine is not available for growth, since D-cystine does not support growth (11) and only the L moiety of meso-cystine is so available.

The cleavage of L-cystathionine under the same conditions was included in the experiment reported in Table II for the purpose of comparing the two isomers which yield cysteine on cleavage. In six experiments in which L-cystathionine was incubated with 5 cc of liver extract, the range of cleavage (judging by Sullivan analyses) was from 29 to 42 per cent (average 33 per cent). Attention is called to the observation that the cleavage of L-cystathionine appears greater when less liver extract is used. In four experiments in which 2 cc of extract were used, the range of cleavage was from 38 to 51 per cent (average 48 per cent).

The presence of desulfurase in liver extracts did not complicate the results obtained on the cleavage of these isomers of L-cystathionine. Desulfurase attacks L-cysteine, producing hydrogen sulfide. The absence of hydrogen sulfide in digests in which homocysteine was produced is consistent with Smythe's observation that desulfurase attacks homocysteine only slightly (5). Apparently D-cysteine is likewise not appreciably attacked by this enzyme, since no hydrogen sulfide was found in digests of D-cystathionine. However, in the case of L-cystathionine hydrogen sulfide tests were always positive when the digestion was carried out in the

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absence of cyanide Under these conditions the presence of hydrogen sulfide indicates the destruction of cysteine formed by the cleavage of L-cystathionine The low yield of cystine in the absence of cyanide may indicate that such destruction is extensive This hypothesis is supported by the observation that the yield of cystine is increased in the presence of cyanide, which protects cysteine from destruction by desulfurase In three experiments in which 2 cc of extract were used in the presence of cyanide the range of cleavage was from 49 to 67 per cent (average 56 per cent)

In the growth studies L-cystathionine had been shown to be capable of supporting the growth of white rats on a cystine-deficient diet (1) *In vitro*, L-cystathionine is cleaved to cysteine On the other hand, L-cystathionine was not able to support growth on a methionine-free diet which contained choline, indicating that this isomer does not yield appreciable amounts of homocysteine *in vivo* In the *in vitro* studies likewise no evidence was obtained that L-cystathionine yields homocysteine Thus, any cleavage to homocysteine can play at best only a small part in the metabolic conversion of L-cystathionine to cysteine

#### SUMMARY

The action of crude liver extract on the isomers of cystathionine has been studied This extract, which has been shown to cleave L-cystathionine, has a minimal action on D-cystathionine under all conditions

L-Allocystathionine is cleaved by the crude liver extract to D-homocysteine The extent of the cleavage approached 100 per cent when high enzyme concentrations were employed Cyanide (0.004 M) was found to inhibit the cleavage

D-Allocystathionine is cleaved by the crude liver extract to D-cysteine The extent of the cleavage was less than in the case of its optical isomer, and the cleavage was inhibited by cyanide

A correlation of the experiments with feeding experiments is presented

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# THE CONVERSION OF THIOCYANATE SULFUR TO SULFATE IN THE WHITE RAT\*

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(Received for publication, June 20, 1947)

The origin and fate of thiocyanate ion in the body have been of interest for more than a century. It has been generally accepted that the ion does not readily permeate normal cell membranes, the distribution of thiocyanate ion in the body is the basis of a method for determination of extracellular fluid volume (1). Despite its assumed extracellular localization, thiocyanate ion is physiologically active. Thus, it may reduce the blood pressure of essential hypertension (2), it may exhibit goitrogenic effects (3), and it may inhibit enzyme systems such as the carbonic anhydrase system (4).

Whether small amounts of thiocyanate ion of endogenous origin are present in the serum and urine of various species has been a matter of some conjecture because methods for its determination in biological fluids have been subject to serious error. It has been demonstrated, however, that ingestion of cyanide ion leads to excretion of increased amounts of thiocyanate ion in the urine (5), and an enzyme system has been isolated which will convert free sulfur and thiosulfate to thiocyanate *in vitro* (6). Intravenous injection of thiosulfate ion as an antidote for cyanide poisoning is well known (7).

In attempting to interpret the physiological activity of thiocyanate ion in terms of its chemical reactivity, we have become interested in the origin and fate of the sulfur portion of the molecule. In this connection, it is of importance to know whether sulfur, once incorporated in the ion, can participate in metabolic interchange with other sulfur-containing compounds. Several mechanisms for sulfur interchange suggest themselves. *In vivo*, thiocyanate ion is readily oxidized to sulfate and cyanide, or is reduced to sulfide and cyanide. In the body such reactions might have little effect on the thiocyanate content if the newly formed cyanide were eventually reconverted to thiocyanate by detoxication. On the other hand, the original thiocyanate sulfur, if released from thiocyanate ion, could be transformed by known pathways of sulfur metabolism. If sulfide ion were formed, it

\* This study was aided by grants from the Rockefeller Foundation and the University of Tennessee Reserve for Research.

might be partially incorporated in tissue protein as cystine which would eventually be converted to sulfate (8) A reaction producing free sulfur would be followed by its oxidation to sulfate (9) Sulfate ion, once formed, should be eventually excreted, although it has been shown that sulfate is deposited in bone to some extent shortly after injection (10) Injected sulfate ion has also been demonstrated to take part in ethereal sulfate synthesis (11) Thus, a means for establishment of the metabolic conversion of thiocyanate sulfur to other sulfur-containing compounds may be looked for in the excretion of extra total sulfate

In order to detect the conversion of administered thiocyanate sulfur to sulfate we have employed isotope tracer techniques Potassium thiocyanate was synthesized from free sulfur containing  $S^{35}$  and potassium cyanide as described in the experimental part The labeled thiocyanate was given intraperitoneally in a single injection to each of three adult albino rats Urine was collected in successive periods of 1, 2, or 3 days and was analyzed for labeled sulfur as total sulfate and as thiocyanate ion, by measuring the radioactivity of isolated samples of these substances At the end of the experimental period, the animals were killed and selected tissues were analyzed for total labeled sulfur Between 1 and 4.5 per cent of the injected thiocyanate sulfur was found in the urine in the form of total sulfate The major part was excreted unchanged and was determined as thiocyanate ion In one instance, the feces were analyzed and were found to contain a total of 5.3 per cent of administered labeled sulfur Urinary thiocyanate and sulfate plus total fecal sulfur amounted to a 91 per cent recovery of injected labeled sulfur No detectable amount of labeled sulfur was found in muscle, adrenal, or thyroid tissue 20 or 25 days after injection of thiocyanate ion, or in bone after 20 days

#### EXPERIMENTAL

*Radioactive Potassium Thiocyanate*—Radioactive barium sulfate<sup>1</sup> was converted to the sulfide by ignition in an atmosphere of hydrogen at  $900^{\circ}$  The sulfide was oxidized to free sulfur with acid iodine solution Potassium thiocyanate labeled with  $S^{35}$  was synthesized by heating the free sulfur with potassium cyanide in acetone according to the method of Castiglioni (12)

Radioactivity measurements were carried out according to the method of Henriques *et al* with the modified Lauritsen electroscope (13) All samples were measured in the form of benzidine sulfate Because of the greater solubility of the benzidine salt, it was recognized that precipitation of the sulfate with barium ion gives greater accuracy for gravimetric determination of daily sulfate excretion Nevertheless, the benzidine precipitate

<sup>1</sup> Radioactive sulfur was obtained from the Clinton Laboratories of the Monsanto Chemical Company at Oak Ridge under allocation from the Manhattan District, Corps of Engineers, in the form of an irradiation unit of potassium chloride

lends itself better to radioactivity measurements and to reprecipitation which is essential to the establishment of the absence of radioactive contaminants in the samples. A 7 mg precipitate was taken as optimum for the measurement of radioactivity in the modified Lauritsen electroscope. The weights of the samples obtained varied from 5 to 20 mg. Corrections were made for background and self-absorption of the sample to correspond to a 7 mg disk of benzidine sulfate, 17 mm in diameter. Aliquot parts were taken to yield activity values at least 10 times the normal background of the electroscope. The results were expressed in micrograms of administered sulfur by comparison of the radioactivity of each sample with that of a standard sample which was prepared from the synthetic potassium thiocyanate after oxidation by the micro-Carius procedure. Standard and unknown samples were measured on the same day.

The radioactive potassium thiocyanate was dissolved in physiological saline for intraperitoneal injection into adult, male, albino rats.<sup>2</sup> The animals were placed in glass metabolism cages (14) and the urine was collected in 15 ml centrifuge tubes. A deflector prevented mixing of the urine and feces. The cage was washed down at the end of each period with water. The washings were added to the urines of Rats 2 and 3 and were preserved separately for Rat 5. In later experiments (Rats 3 and 5), a drop of liquefied phenol was added to the collection tube to prevent bacterial action. At the end of each collection period, the urine was placed in the refrigerator.

*Sulfate-sulfur determinations* were carried out by the method of McKitttrick and Schmidt (15). In order to obtain quantitative recovery from urines to which known amounts of radioactive sulfate had been added, some modifications of the method had to be introduced. The thiocyanate was precipitated from the urine samples from Rat 2 or 3 by addition of an excess of 2 per cent cuprous chloride in 0.6 N hydrochloric acid. The cuprous thiocyanate was separated by centrifugation and decantation, washed once with water, and preserved for determination of thiocyanate sulfur. The supernatant and washings were made up to volume for total sulfate determination. Hydrolysis of ethereal sulfates and removal of phosphate were carried out as described by Fiske (16). This treatment also removed excess copper ions.

To 10 ml of the dephosphated solution of total sulfate were added 3 drops of 10 per cent Triton<sup>3</sup> wetting agent, 1 drop of 0.5 per cent bromophenol

<sup>2</sup> The specific activity of the administered thiocyanate sulfur varied with the radioactive decay of the samples. No data are available to estimate the absolute activity of the samples in terms of radiation units. In these experiments a 10<sup>4</sup> dilution of the injected sulfur was measurable at a level of 10 times the background of the instrument used.

<sup>3</sup> Triton NE was supplied by the Rohm and Haas Company.

blue solution, and 2 ml of  $N$  sodium acetate. Enough hydrochloric acid was added to change the color of the indicator to yellow. The volume was adjusted to 15 ml and 15 ml of 0.09  $N$  solution of benzidine in 0.4  $N$  hydrochloric acid were added slowly. After 10 minutes, 10 ml of acetone were added. After standing from 18 to 24 hours, the solution was filtered to collect the precipitated benzidine sulfate on a paper disk. The precipitate was washed with 95 per cent acetone. The sample was dried at room temperature over phosphorus pentoxide and weighed. Radioactivity measurements were made with the Lauritsen electroscope, and then the pad of benzidine sulfate was dissolved in 2 ml of  $N$  ammonium hydroxide with warming. A drop of bromophenol blue indicator solution, 2 drops of 10 per cent Triton wetting agent, 1 ml of 1 per cent potassium thiocyanate solution, and 1 ml of  $N$  acetic acid were added. The solution was adjusted with hydrochloric acid to the bromophenol blue end-point and was diluted to 5 ml. The sulfate was precipitated with 2.5 ml of benzidine reagent and 2 ml of acetone, and was collected, dried, and weighed. If the radioactivity measurement on the reprecipitated sample did not agree with the value obtained for the original, the sample was again dissolved and precipitated. In these experiments, however, one reprecipitation sufficed.

*Thiocyanate-sulfur determinations* were performed on the cuprous thiocyanate precipitates which had been removed prior to total sulfate analysis of the urine. The precipitate was digested with 1 ml of 20 percent sodium carbonate solution for 20 minutes on the water bath. The supernatant liquor was collected, combined with washings of the residue, and an aliquot part was taken. This was acidified strongly with hydrochloric acid, warmed, and oxidized with potassium biomate to convert the thiocyanate to sulfate and cyanide. Some inactive sodium sulfate was added at this point to assure a benzidine sulfate precipitate weighing as much as 7 mg. This solution was evaporated to dryness and the residue was taken up in water. The sulfate was precipitated as the benzidine salt and radioactivity determinations were done in the manner described for total sulfate.

The amounts of cuprous thiocyanate precipitate formed by this method in 24 hour samples of the urine of Rats 2 and 3 were very small. It was found that recovery of thiocyanate ion added in comparable amounts to normal rat urine was not more than 60 per cent. Accordingly, an improved method for determination of labeled thiocyanate sulfur in urine was devised for the experiment with Rat 5 as follows. To an aliquot part of urine (usually 3 ml) was added an amount of inactive potassium thiocyanate estimated to be more than 100 times the labeled thiocyanate present. Silver acetate in 3  $M$  ammonium hydroxide was added until complete precipitation of silver salts resulted and the pH was adjusted with acetic acid until the solution was just acid to litmus paper. The silver



salts were separated by centrifugation and were washed with water. The supernatant liquor and washings were freed of silver ion with hydrochloric acid. This solution was set aside for determination of the total sulfate as described above.

A random portion of the mixed silver salts was suspended in 3 ml of water, and 2 ml of 5 M hydrochloric acid and 50 mg of potassium biomide were added. The solution was warmed nearly to boiling and was titrated with 0.01 N potassium bromate with methyl red as an indicator. The sulfate content of the solution was calculated from the equivalence of the bromate used and the radioactive sulfate was determined in the manner described for total sulfate. Control titrations of known amounts of silver thiocyanate under these conditions showed that the bromate titer was a quantitative measure of the thiocyanate which was oxidized to sulfate. The amount of total labeled thiocyanate ion in the urine was then calculated by means of the isotope dilution method, assuming that the mass of the original urinary thiocyanate ion could be neglected in relation to the 100-fold excess of inactive carrier.

The silver salt method of partition of sulfate and thiocyanate sulfur was used on acid extracts of the feces of Rat 5. Unlike the silver salts precipitated from the urine, those precipitated from normal feces included considerable sulfur-containing material which was oxidizable with biomate. Therefore, the "thiocyanate sulfur" determined for feces represents total unoxidized sulfur compounds which are precipitable by silver ion.

*Total labeled sulfur* was determined on organs, tissues, and feces by the method of Bailey (17).

#### DISCUSSION

A conclusion that the labeled sulfate sulfur found in the urine (Table I) represents an actual metabolism of a small amount of thiocyanate ion requires careful consideration of the factors involved in these experiments. Demonstration of the absence of radioactive contaminants in the samples on which radioactivity measurements are made is of first importance in tracer experiments. Here the sulfate was precipitated as the benzidine salt from solutions known to contain other radioactive substances. Control experiments had shown that significant amounts of radioactivity did appear in samples of benzidine sulfate precipitated in the presence of extremely small concentrations of radioactive thiocyanate ion. This contaminating radiation could not be entirely eliminated by adding large amounts of inactive thiocyanate ion to the solution before precipitation. We therefore set up the following precautions for precipitating total sulfate samples in the presence of other compounds containing radioactive sulfur. Thiocyanate ion in the urine was diluted with additional inactive thiocyanate and then

TABLE I  
*Urinary Excretion of Labeled Sulfur after Injection of Potassium Thiocyanate*

Day	Urinary SO <sub>4</sub> S excretion	Labeled S as SO <sub>4</sub> S	Labeled S as SCN S	Day	Urinary SO <sub>4</sub> S excretion	Labeled S as SO <sub>4</sub> S	Labeled S as SCN S
1	mg	γ	γ	1	mg	γ	γ
2	7 65	69 5	4690	2	14 7		
3	9 45			3	8 9		
4	9 04	9 2	134	4	8 2		
5	15 9	0 38	7 5	5	13 0		1890
6	17 8	5 3	5 2	6			
	12 2	1 1	11 8	7			
Total		85 5		8			
1-2	12 1			9-11			
3-4	13 7			12-14			
5-6	15 8	27 5	633	15-17			
7-8	17 7	18 6	20 6	18-20			
9-10	32 8	14 5	5 62	21-23			
11-12	12 5	4 1	3 47	4-8			
13-14	15 7	3 9	3 98	9-23			
15-17	13 8	3 2	1 32	4-23			
18-20	20 1	3 1	1 24				
21-23	10 2	1 2	0 41				
24-27	10 9	†	0 24				
28-29	9 5	(0 3)†	0 15				
Total		76 4					

\* Diet 1, sucrose 24, starch 24, casein 18, Mazola oil 3, lard 10, cod liver oil 2, yeast 15, salt mixture 4 (Hawk and Oser)

† The radioactivity measured was less than 10 times the background

removed as completely as possible with cuprous or silver ion. Any radioactive thiocyanate remaining in solution was again diluted with inactive thiocyanate before precipitation of the sulfate with benzidine. Finally, after a quantitative measurement of the radioactivity was obtained, the benzidine sulfate was dissolved and reprecipitated in the presence of more inactive thiocyanate. Radioactivity measurements on the second precipitation, to be considered as representing radioactive sulfur in the form of sulfate, were required to agree with those found on the first. With this method, control experiments showed that the inactive sulfate solutions contaminated with radioactive thiocyanate could be precipitated as the benzidine salt entirely free of the added radioactivity. It thus appears that our radioactivity measurements actually represent labeled sulfate present in the samples of urine and feces.

The site and mechanism of the oxidation of thiocyanate sulfur to sulfate cannot be deduced from these experiments. The possibility that the small amount of oxidation of thiocyanate sulfur found in these experiments was produced by bacteria in the intestine, or in the voided urine, cannot be entirely discounted. In our experiments with Rats 3 and 5, bacterial action in the collected urine was prevented by the addition of phenol. Of the feces sulfur, the thiocyanate plus sulfate was about one-third of the total labeled sulfur present, which suggests the presence of transformation products of thiocyanate ion in the feces. Stuber and Lang (18) were unable to demonstrate destruction of thiocyanate incubated with intestinal contents, although it is questionable whether their methods would detect the order of magnitude of oxidation found in the present study. The unidentified sulfur in the feces amounted to 4 per cent of the injected, labeled sulfur. This was a small fraction in an experiment in which the total recovery of thiocyanate plus sulfate was 87 per cent.

Analyses of muscle, adrenals, and thyroid from Rats 3 and 5 indicated no selective immobilization of labeled sulfur had occurred in these tissues. Likewise, no deposited radioactive sulfur was detected in the bones of Rat 5.

It seems possible that a spontaneous oxidation of thiocyanate sulfur to sulfate can account for the formation of sulfate in very dilute solutions of thiocyanate ion within the animal organism. We observed that the most dilute solutions of potassium thiocyanate for colorimetric standards, kept in diffuse light in clear, glass-stoppered bottles, gradually lessen in their color values. Using the sensitive isotope method of analysis we have found that a solution of 5.1  $\gamma$  of potassium thiocyanate per ml of ordinary distilled water was 42.5 per cent converted to sulfate in 45 days and was 61 per cent converted in 76 days, while a more concentrated solution of 10 mg of potassium thiocyanate per ml of distilled water had been oxidized to

sulfate only to the extent of 12 per cent in 30 days. This result might arise from primary oxidation of thiocyanate to thiocyanogen, which is analogous to the spontaneous oxidation of iodide to free iodine. Free thiocyanogen thus formed in water solution would decompose to sulfuric acid and hydrocyanic acid (19). This factor certainly contributes to the occurrence of sulfate in urines voided many days after the injection of the labeled thiocyanate sulfur. It doubtless accounts, also, for the high ratio of sulfate to thiocyanate found in the final cage washings of Rat 5 (Table I), since 63 days elapsed before this sample was analyzed.

Whether the mechanism of the *in vivo* conversion of thiocyanate ion to urinary sulfate is spontaneous or metabolic, these experiments show that relatively large amounts of thiocyanate ion in the body produce very little sulfate. In consequence, it appears that very little, if any, of administered thiocyanate ion is metabolized by the organism.

#### SUMMARY

The excretion of sulfate and thiocyanate ion in the urine after injection of potassium thiocyanate labeled with radioactive sulfur has been studied. Between 1 and 4.5 per cent of the injected thiocyanate sulfur appeared as sulfate, the major part was excreted unchanged. Several possibilities for the conversion of thiocyanate to sulfate in the animal body and excreta are discussed.

No accumulation of labeled sulfur was found in the thyroid, adrenals, or muscle after 20 or 25 days. None was found in the bones after 20 days. About 5 per cent of the sulfur of injected thiocyanate was found in the feces.

Factors involved in the use of the benzidine sulfate method for urinary radioactive sulfate are discussed. A method for the determination of ultramicro quantities of labeled thiocyanate ion in urine is described.

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# FACTORS AFFECTING THE GROWTH OF RATS RECEIVING NIACIN-TRYPTOPHAN-DEFICIENT DIETS\*

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The growth-retarding effect of corn products on animals receiving low protein diets has given rise to a number of postulated mechanisms for this phenomenon and for its reversal by niacin or tryptophan (1-4). The growth-suppressing effect of supplements other than corn (2) and the suggestion that amino acid imbalance can explain this effect (5) have been extended to the chick (6). That tryptophan is involved in niacin synthesis is indicated by the results of excretion studies upon administration of tryptophan to the rat (7, 8), the horse and the cotton rat (9), and the human (10). Investigations on mutant strains of *Neurospora* have revealed several genetically distinct strains requiring niacin, but none of those studied could use tryptophan to replace niacin (11).

A relationship more involved than a synthesis of niacin from tryptophan in the animal tissues is indicated by the profound effect of the type of carbohydrate in the diet (12) and by the effectiveness of certain proteins, protein hydrolysates, and crystalline amino acids in producing the deficiency state (5, 6).

The results reported here represent an extension of the work dealing with the growth-suppressing action of amino acids, proteins, and acid-hydrolyzed proteins. Additional studies indicate that, under our experimental conditions, indole-3-acetic acid, indole-3-acrylic acid, and indole-3-butyric acid do not exert any effect on the growth of rats when incorporated into 9 per cent casein-sucrose diets at levels comparable to those used by Kodicek *et al* (4). These workers reported that indole-3-acetic acid suppressed the growth of rats in the same manner as corn grits. A mixture of crystalline

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the National Dairy Council, on behalf of the American Dairy Association, and the Nutrition Foundation, Inc.

We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the supply of crystalline B vitamins, to the Abbott Laboratories, North Chicago, Illinois, for the generous supply of haliver oil, and to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for the synthetic *Lactobacillus casei* factor.

amino acids simulating 2 per cent acid-hydrolyzed casein does suppress the growth of niacin-deficient rats receiving a marginal level of tryptophan

#### EXPERIMENTAL

The experimental procedure used was the same as in previous studies (1, 2, 5) Sprague-Dawley, 3 week-old weanling rats kept in individual cages, were fed the diets *ad libitum* for a 5 week experimental period, weighings were made at weekly intervals. The basal diet had the following composition: sucrose 81.8 per cent, casein<sup>1</sup> 9 per cent, Salts IV (13) 1 per cent, corn oil 5 per cent, L-cystine 0.2 per cent, and vitamins added as a dry mixture diluted with sucrose to provide the following quantities, per 100 gm of diet, thiamine 0.2 mg, riboflavin 0.3 mg, pyridoxine 0.25 mg, *dl*-calcium pantothenate 2 mg, choline chloride 100 mg, inositol 10 mg, biotin 0.01 mg, and folic acid 0.02 mg. Fat-soluble vitamins were provided in the form of halibut liver oil fortified with vitamins E and K and diluted with corn oil. This was fed by dropper once weekly at a level sufficient to provide approximately the following daily intakes per rat: vitamin A 6 i.u., vitamin D 0.06 i.u., 2-methyl-1,4-naphthoquinone 0.04 mg,  $\alpha$ -tocopherol 0.5 mg.

The rate of growth of the rats on the basal ration varied from one experiment to another. The average gains of three control rats in a different series of experiments ranged from 7.8 to 15.7 gm per week with a variation of 4.8 to 18.6 gm for individual animals in all groups. Variation in groups from 10.4 to 20.8 gm and individual variation from 9.8 to 22.8 gm per week were noted in the groups receiving added niacin at a level of 1.5 gm per 100 gm of diet.

Because of this variation, negative control groups (no niacin) and positive control groups (added niacin) were included in every experiment. The average growth of twenty-four negative control rats was 11.1 gm per week and of twenty-four positive control animals 16.1 gm per week in eight series of experiments. The growth data obtained on supplementing the basal ration are presented in Table I. The tabulation of the rate of growth as percentage of negative control groups when no niacin was given, and as percentage of growth of positive control groups when the experimental groups were supplemented with niacin or tryptophan, permits the averaging of values for similar groups in different experiments.

With this technique a large number of substances were tested for their ability to induce niacin deficiency in rats. The criteria used for these tests were (1) the substance must suppress growth at the level fed to 60 per cent or less of the growth of the negative control group, and (2) the addition of niacin or tryptophan must result in growth of more than 90 per cent of the

<sup>1</sup> Smaco purified casein



TABLE I

*Growth-Depressing Effect of Certain Supplements to Low Niacin Diet Containing Sucrose and 9 Per Cent Casein*

Supplement	Without niacin		Plus 1.5 mg niacin per 100 gm ration		Plus 20 mg DL tryptophan per day	
	No of animals	Average gain as per cent of negative controls*	No of animals	Average gain as per cent of positive controls*	No of animals	Average gain as per cent of positive controls*
1% glycine	9	86	3	98		
2% "	18	38	9	101	3	152
2% " + 1% sodium benzoate	3	58				
2% glycine + 2% sodium benzoate	3	43				
2% glycine + 3% sodium benzoate	9	104				
3% sodium benzoate	6	-30				
2% glycine + 2% casein	3	75	3	191		
2% " + 1% L-proline	3	36				
3% gelatin	6	49				
6% "	3	45				
2% casein	3	169	3	127		
2% acid-hydrolyzed casein	6	31	6	100		
2% " fibrin	3	10				
2.4% amino acid mixture†	6	38	4	101	1	126
2.4% " " " less DL alanine and glycine	3	37	1	162	1	129
1% L arginine hydrochloride	3	126				
1% DL-alanine*	3	61				
2% "	6	118	3	124		
2% L leucine	3	50				
2% sodium pyruvate	3	101	3	85		
2% " glycolate	3	109	3	92		
2% " lactate	3	116				
1% urea	3	130				
1% NH <sub>4</sub> Cl	3	126				
1.5 mg % indole-3-acetic acid	6	124	6	118	3	112
3 " % " "	3	103	3	118		
5 " % " "	3	143	3	128		
1.5 " % indole-3-acrylic "	3	182				
5 " % " "	3	96				
1.5 " % indole 3-butyric acid	3	129				
5 mg % " "	3	103	3	142		

\* See the text

† Mixture of crystalline amino acids (made to simulate 2 per cent acid hydrolyzed casein), 2.4 gm added to each 100 gm of diet contained L arginine hydrochloride 0.082 gm, L histidine hydrochloride H<sub>2</sub>O 0.050 gm, L lysine hydrochloride H<sub>2</sub>O 0.138 gm, L tyrosine 0.128 gm, DL phenylalanine 0.104 gm, L cystine 0.007 gm, DL serine 0.120 gm, DL threonine 0.156 gm, L leucine 0.242 gm, DL isoleucine 0.260 gm, DL-valine 0.280 gm, L glutamic acid 0.456 gm, DL aspartic acid 0.126 gm, glycine 0.010 gm, and DL alanine 0.112 gm

positive control group This means that the growth must be less than 6.7 gm per week without niacin and at least 14.5 gm per week when niacin is added, before any growth depression can be considered as an induced niacin deficiency

It will be noted (Table I) that 2 per cent glycine, 2 per cent acid-hydrolyzed casein, and 2.4 per cent amino acid mixture, the equivalent of 2 per cent acid-hydrolyzed casein with or without glycine and alanine, were the only supplements fed which induced a niacin deficiency Other supplements, *viz* 2 per cent glycine plus 1 per cent proline, 3 per cent gelatin, 6 per cent gelatin, 2 per cent acid-hydrolyzed fibrin, and 2 per cent L-leucine, also depressed growth to a sufficient extent to fulfil the first criterion, but no corresponding groups were given niacin supplementation Previous work (2, 5), however, has shown that the depression of growth on some of these supplements is reversed by niacin or tryptophan A number of individual amino acids and simple compounds related to glycine were fed without any evidence of growth-suppressing action (Table I)

Rats receiving the 2 per cent glycine supplement failed to gain beyond 60 to 80 gm, and eventually lost weight and died if they were not given niacin or additional tryptophan The hair coat became rough and some hair loss occurred, but no other abnormalities were noted

Rats fed the basal ration supplemented with 3 per cent sodium benzoate lost weight and showed evidence of toxemia When, in addition to the sodium benzoate, 2 per cent glycine was present in the ration, growth was restored to that found in the absence of both supplements Since the conjugation to form hippuric acid is thought to occur in the liver, these results might be interpreted as indicating that the glycine exerts its growth-suppressing effect on a low protein, niacin-free diet systemically and not in the intestinal tract

Several attempts were made to induce growth retardation by feeding low levels of indole-3-acetic acid (m.p. 166-168°) and related auxins It is evident from the data in Table I that no inhibition was observed In most cases, growth was slightly greater than that in corresponding control groups

In studies with diets containing corn grits, Krehl *et al* (15) showed that niacin and tryptophan act additively in supporting improved growth Similar work with the diet used here, plus 2 per cent glycine, indicates that, while added tryptophan improved the response to low levels of niacin, niacin did not improve the response to the addition of 10 and 20 mg of DL-tryptophan per 100 gm of ration (Table II) This apparent contradiction might be due either to excess tryptophan or inadequate niacin to show the additive effect

A further attempt to bring out the metabolic relationship between

TABLE II

*Effect of Niacin and Tryptophan in Promoting Growth of Rats Receiving 9 Per Cent Casein Sucrose Diet with 2 Per Cent Added Glycine*

Dl. Tryptophan	Niacin	Weekly gain	
		Average of 3 rats	Range
mg per cent	mg per cent	gm	gm
0	0	3 3	1 6- 4 8
0	0 1	5 3	3 0- 7 4
0	0 2	6 6	3 6- 9 6
0	0 5	9 0	6 6-11 6
0	1	13 0	11 5-15 2
0	1 5	15 8	12 4-19 4
10	0	8 7	5 4- 9 0
10	0 1	8 1	3 8-10 6
10	0 2	9 8	6 8-11 4
20	0	10 7	10 0-11 2
20	0 1	10 8	9 6-12 0
30	0	11 0	6 4-14 0
50	0	11 9	7 6-16 4
100	0	12 5	9 6-15 6

TABLE III

*Supplementary Effect of Niacin and Tryptophan in Promoting Growth of Rats Receiving 12 Per Cent Acid Hydrolyzed Casein\*-Sucrose Diet*

Supplement		Weekly gain	
		Average of 3 rats	Range
		gm	gm
50 mg %	tryptophan	0 6	0 - 1 2
100 "	" "	6 0	4 8- 7 0
150 "	" "	12 0	11 -14 6
50 "	" " + 1 5 mg % niacin	1 1	0 6- 1 4
100 "	" " + 1 5 " % "	9 0	5 5-14 0
150 "	" " + 1 5 " % "	10 5	6 8-13 6

\* Smao casein was hydrolyzed with 10 volumes of 3 N H<sub>2</sub>SO<sub>4</sub> for 24 hours at 15 pounds pressure in the autoclave and neutralized with barium hydroxide. The barium sulfate was removed by filtration and washed three times with hot water. The combined filtrate and washings were concentrated *in vacuo* to a syrup which was dried to a porous cake in a vacuum oven at 50°. The mixture was finely powdered in a mortar and stored in a brown glass bottle until it was incorporated into the dry ration. The mixture was light amber, very hygroscopic, and gave a negative biuret test.

tryptophan and niacin was made by feeding rats a tryptophan- and niacin-deficient diet with 12 per cent acid-hydrolyzed casein supplemented with

0.6 per cent methionine and 0.2 per cent cystine. The addition of graded quantities of tryptophan to the diet with and without niacin permitted growth proportional to the tryptophan fed. The presence of niacin appeared to have a slight, if any, growth-augmenting effect (Table III).

With dextrin (cooked starch) replacing sucrose as the carbohydrate (Table IV), 2 per cent glycine and 4 per cent glycine did not induce a niacin deficiency. 4 per cent glycine addition did suppress growth, but niacin had no beneficial effect. 2 per cent succinylsulfathiazole in addition to 2 per cent glycine was without effect. That this failure to observe growth depression is not due to the niacin provided by the dextrin is indicated by analyses of the dextrin-containing ration (12). In earlier work (2) 6 per cent gelatin did induce growth suppression, counteracted by niacin or tryptophan.

TABLE IV  
*Growth of Rats on 9 Per Cent Casein-Dextrin\* Rations*

Supplement	No. of rats	Average weekly gain
		gm
None	15	20.1
1.5 mg % niacin	12	17.8
2 % glycine	9	18.7
2 % " + 1.5 mg % niacin	6	18.7
2 % " + 2 % succinylsulfathiazole	3	18.6
4 % glycine	3	12.6
4 % " + 1.5 mg % niacin	3	12.5

\* Prepared by autoclaving raw corn starch with sufficient water to give a thick paste for 3 hours at 15 pounds pressure.

From the results shown in Table I it is evident that the effect of free amino acids, whether fed as a casein hydrolysate or as a mixture of crystalline amino acids, is the same. That these amino acids or the casein hydrolysate are non-toxic in the presence of adequate niacin is evident from the data given here and from unreported experiments in this laboratory, wherein growth rates of approximately 20 gm per week have been noted with a mixture of crystalline amino acids as the sole source of nitrogen.

#### DISCUSSION

Salmon (14) has recently reported poor growth, correctable by niacin, in rats receiving a diet low in fat containing up to 18 per cent casein and no known source of a deleterious agent. Corn-meal or corn grits did not depress growth but resulted in improvement when fed with 9 per cent casein. This is not unexpected, since the growth was less than 3 gm per

week without corn products in the diet. This represents a rate of growth comparable to that previously reported for rats receiving corn grits (1, 2). Salmon's diet differs from the one used in these studies chiefly in that it contains a lower level of fat and the casein was from a different source.

In our recent work, we have found small but quite consistent differences between the growth of negative and positive control groups, but growth has always been depressed much below negative controls by the supplements listed above. Salmon was able to prevent the growth depression from lack of niacin by increasing the fat content of his ration to 30 per cent. This might be interpreted as further evidence that the intestines are a site of synthesis of significant quantities of available niacin, since the level of fat in diets is known to affect riboflavin synthesis in the tract of rats (16).

Attempts to explain the niacin-tryptophan relationship have centered around (1) synthesis of niacin in tissues from tryptophan or (2) synthesis in the gastrointestinal tract by microorganisms. From the variety of types of experimental evidence on this subject, with frequent apparent contradictions, it would appear that both types of synthesis may occur. The experimental conditions employed, the species, strain, and age of the animal, and other uncontrolled factors are probably important in niacin metabolism (17).

Evidence for the manner in which free amino acids exert their detrimental effect under these experimental conditions is lacking. It seems unlikely that these substances could exert this effect anywhere except in the intestinal tract, since the quantities fed in free form represent only 20 to 25 per cent increase in the total amino acid intake. That free dietary amino acids affect the type, quantity, or location of intestinal microorganisms is an attractive possibility. The site of synthesis of niacin by such organisms (18) probably is a major factor in determining its availability to the host animal.

#### SUMMARY

1 Growth suppression of rats receiving a 9 per cent casein-sucrose, niacin-free diet was noted upon supplementation with 2 per cent glycine, 2 per cent acid-hydrolyzed casein, or the crystalline amino acids contained in 2 per cent acid-hydrolyzed casein.

2 The growth-depressing effect of glycine could not be shown on a diet with dextrin as the source of carbohydrate.

3 Indole-3-acetic acid and related compounds exerted no growth-depressing effect under our experimental conditions.

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# BIOCHEMISTRY OF THE SPHINGOLIPIDES\*

## II ISOLATION OF DIHYDROSPHINGOSINE FROM THE CEREBROSIDE FRACTIONS OF BEEF BRAIN AND SPINAL CORD

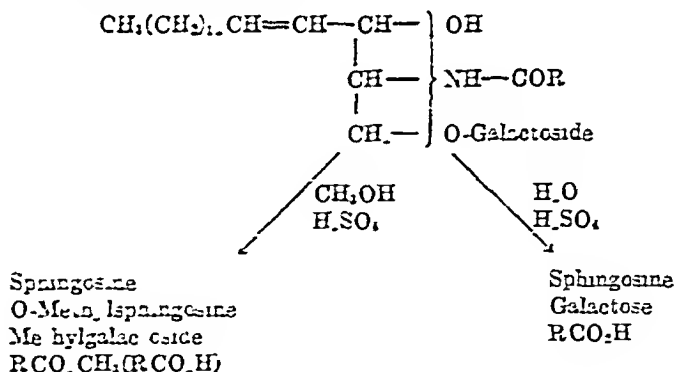
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(Received for publication, April 23, 1947)

We recently described a practical method for the large scale isolation of sphingolipides from beef brain and spinal cord (2). The present paper deals with the preparation of sphingosine and dihydrosphingosine from such material.

Cerebrosides are hydrolyzed readily by aqueous sulfuric (3-5) or hydrochloric (6) acid, by methanol or ethanolic-sulfuric acid (4, 6, 7), and by methanolic-hydrogen chloride (8).



\* The authors wish to express their deep appreciation to The Upjohn Company for a fellowship in support of this work.

The material presented in this paper was taken from the theses submitted by F. J. Glick, W. P. Norris, and G. E. Phillips to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in Chemistry. A preliminary report of a portion of this work has previously been published (1).

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in about the same proportion as did crude sphingolipide. As a final point in this connection it should be noted that neither free sphingosine nor sphingosine sulfate is easy to purify or identify. For this reason sphingosine has been characterized generally as the triacetyl derivative. Unfortunately triacetyldihydrosphingosine melts at almost the same temperature as does triacetylsphingosine and gives very little depression of melting point on mixing. Optical activity provides a precise method of differentiation since the two derivatives rotate in opposite directions, but such determinations were not generally made in the early work in this field, and it seems possible that some of the products described in the literature may actually have consisted of mixtures of sphingosine and dihydrosphingosine.<sup>1</sup> The importance of this point is illustrated in Section IV, C. Derivatives of sphingosine and dihydrosphingosine may also be distinguished by iodine number determinations (see Table II). However, the results are not clean-cut since the double bond in sphingosine does not react quantitatively with the usual reagents. For example, pure triacetylsphingosine gave an iodine number of 52.7 as compared with a theoretical value of 59.7.

In order to characterize sphingosine and dihydrosphingosine further the N-benzoyl and tribenzoyl derivatives were prepared. Tribenzoyldihydrosphingosine (m.p. 144–145°) is an excellent derivative for purification and identification purposes. It crystallizes beautifully from ethanol and is purified readily from that solvent. We have never been able to obtain pure triacetylsphingosine. The various samples melted over a 2–3° range between 118–124° (Reichel and Thannhauser (14) prepared tribenzoylsphingosine, melting at 118–120°). It is possible that cis-trans isomerism is responsible for the difficulty. Niemann (8) has reported two isomers of sphingosine in methanolic-hydrogen chloride hydrolysates of sphingolipides. This question will be discussed in a future publication.

The N-benzoyl derivatives of sphingosine and dihydrosphingosine have not been obtained in crystalline form. They tend to form stiff gels from a variety of solvents and, on drying, yield amorphous powders. N-Acetyldihydrosphingosine is the only compound of this type we have been able to obtain in a crystalline state.

The isolation of dihydrosphingosine from cerebrosides of beef brain and spinal cord raises a number of interesting questions regarding its possible occurrence in sphingomyelin and other sphingolipides, its distribution in sphingolipides of other organs and of other species, its variation with the

<sup>1</sup> Kitagawa and Thierfelder (9) described a base sulfate which was insoluble in hot alcohol. Later Thomas and Thierfelder (12) prepared a triacetyl derivative melting at 100–102° from this material and commented that the derivative melted slightly higher than triacetylsphingosine. Klenk and Diebold (4) reported a triacetyl derivative melting at 97–98° which they could not purify further. It seems possible that both of these products contained triacetyldihydrosphingosine.



age of the animal, and its metabolic relationships to sphingosine. Data on certain of these problems will be reported in subsequent papers of this series.

From a preparative standpoint the presence of dihydrosphingosine complicates considerably the isolation of pure sphingosine. We have not been able to separate the two bases cleanly by fractionation of either the free bases or their sulfates. Furthermore, in order to obtain pure triacetyl-sphingosine from a mixture containing any considerable proportion of the dihydro base, it was necessary to take special precautions in fractionation of the sulfates and the N-acetyl derivatives. In this connection it should be noted that the lower content of dihydrosphingosine in brain than in spinal cord makes the former tissue the preferable starting material for the preparation of sphingosine, despite the lower yields of sphingolipide obtained from it.

## EXPERIMENTAL

### *I Hydrolysis of Sphingolipides with Methanolic-Sulfuric Acid*

100 gm of sphingolipide were added to a solution of 112 ml of concentrated sulfuric acid in 2300 ml of methanol, and the mixture was heated on the steam cone under a reflux for 5 to 6 hours<sup>2</sup>. The methanol solution was cooled in an ice bath and the precipitated fatty acids and methyl esters were removed by filtration. The filtrate was extracted four times with 1 liter portions of low boiling (30–60°) petroleum ether to remove the remainder of the fatty acids and esters. The methanol solution was freed of petroleum ether by concentration on the water pump, and the sulfuric acid was neutralized (to phenolphthalein) with 4 N methanolic potassium hydroxide solution (660 to 700 ml required). The precipitated potassium sulfate was removed by filtration with a filter aid. The filtrate<sup>3</sup> was made acid to litmus with glacial acetic acid (40 to 50 ml) and concentrated to 300 to 500 ml on the water pump. The solution was then made strongly alkaline to phenolphthalein and the bases were extracted with two 1 liter portions of ether. The ether extracts were combined and washed carefully with cold water<sup>4</sup>. The solution was dried over sodium sulfate and

<sup>2</sup> With crude brain sphingolipide a dark brown solution resulted at this point, whereas spinal cord material darkened only slightly, and recrystallized cerebrosides gave an almost colorless, water-clear solution.

<sup>3</sup> If this solution is allowed to stand in the ice box overnight, a crystalline solid separates. This material appears to consist mainly of potassium methyl sulfate, which explains the fact that only about 70 per cent of the expected amount of potassium hydroxide was required to neutralize the sulfuric acid.

<sup>4</sup> Intractable emulsions tend to form at this point, especially if more than two washings are made. Apparently material soluble in a methanol ether mixture precipitates as the methanol is removed by the water. It should be noted that spinal cord material caused much more trouble than brain at this point.

the ether was removed, giving a crude "sphingosine base" fraction. The yield varied from 16 to 28 gm (theoretical yield from 100 gm of cerebroside, 36 gm). Crude brain sphingolipide gave the poorest yield (Table I, C) and the product was a dark brown wax. Crude spinal cord sphingolipide gave a somewhat better yield of a light brown solid (Table I, A). Purified cerebroside fractions obtained by recrystallizing crude material from 10 volumes of glacial acetic acid gave the best yields and the products were almost white, friable solids (Table I, B and D).

TABLE I  
Yield of Base Sulfate Fractions from 100 Gm Portions of Sphingolipide

Source and nature of sphingolipide used	Base sulfate (gm per 100 gm sphingolipide)				
	Total	Fraction I	Fraction II		Fraction III
			(a)	(b)	
A Spinal cord, crude, 2nd alcohol extract	25	5.0 (180-210°)*	5.0	6.0	4.5
B Same as "A," except recrystallized from acetic acid	28	7.0 (190-220°)	6.0 (180-210°)	6.0 (140-220°)	6.0 (70-150°)
C Brain, crude, 2nd alcohol extract	16	1.4	4.0	4.0	3.0
Same as "C," except recrystallized from acetic acid	23	2.0	13.0		4.0
E Spinal cord, recrystallized from acetic acid	22	4.0	11.0		
F Brain, recrystallized from acetic acid	18	1.8	8.0		4.0
G Spinal cord, 1st alcohol extract	23	6.6	11.0		4.0

\* The figures in parentheses are decomposition points

## II Preparation and Fractionation of Base Sulfates

The crude base fraction (20 to 28 gm) was dissolved in 10 volumes of absolute ethanol and 1 N ethanolic-sulfuric acid (27.7 ml of concentrated sulfuric acid diluted to 1 liter with absolute ethanol) was added to the first faint blue with Congo red.<sup>5</sup> The mixture was cooled in an ice bath and the precipitated sulfate mixture was removed by filtration,<sup>6</sup> washed with

<sup>5</sup> It is essential to stop at this point, since the precipitated sulfates redissolve in an excess of sulfuric acid (2, 3)

<sup>6</sup> The filtrate, on concentration *in vacuo*, yields a small quantity of hygroscopic, sticky material from which little sphingosine can be obtained

a small volume of cold ethanol, and dried *in vacuo* over phosphorus pentoxide, giving 17 to 25 gm of material which was white to light brown in color. The crude sulfite mixtures were fractionated from methanol as follows: a 25 gm portion was heated on the steam cone with 1800 to 2000 ml of boiling methanol. The brain product dissolved almost completely, the spinal cord material left a considerable insoluble residue. The mixture was cooled to room temperature and the solid filtered (Fraction I). This material is a stable, friable, non-hygroscopic solid melting with decomposition around 200°. The filtrate was concentrated to 300 to 400 ml *in vacuo* and cooled in an ice bath, yielding Fraction II. In some experiments Fraction II was divided into Fraction II, *a* (precipitated at room temperature) and Fraction II, *b* (precipitated by cooling the filtrate from Fraction II, *a* in an ice bath). These fractions were usually crystalline, white, slightly hygroscopic solids which slowly turned brown on standing exposed to air.

The filtrate from Fraction II on concentration and cooling yielded additional fractions of hygroscopic solid from which a poor yield of sphingosine derivative was obtained (see Section III, B, Fraction G, III). This material (Fraction III) presumably contained the O-methyl ethers. The data on several runs are summarized in Table I.

Each of the fractions (Nos. I, II, III) melted with decomposition over a wide range, failed to redissolve completely in a volume of solvent in which it originally was soluble, and on repetition of the fractionation process could be separated into two or three fractions. Since recrystallization from methanol (and ethanol) did not yield pure substances, other solvents were tried. Of these only acetic acid gave promising results. Fraction B, I (2.0 gm) was recrystallized from 200 ml of hot glacial acetic acid, yielding 1.4 gm of nicely crystalline material melting at 190–230°. A second crystallization from 200 ml of acetic acid gave 1.3 gm, m.p. 200–240°. However, even this purified dihydrosphingosine sulfate still contained a small proportion of other material, as judged by the properties of the triacetyl derivatives.

### III Preparation of Triacetylsphingosine

Pure triacetylsphingosine (m.p. 101–102°,  $[\alpha]_D^{25} = -11.7^\circ$  (0.1 gm in 10 ml of chloroform)) can be obtained directly from the unfractionated sulfates prepared from brain sphingolipides. Apparently the dihydrosphingosine content is so small that it does not interfere seriously with the isolation of the triacetyl derivative. The behavior of the spinal cord sulfate is markedly different. It is difficult to obtain pure triacetylsphingosine from this material unless special attention is paid to its fractionation. Fraction I from spinal cord contains 40 to 60 per cent of dihydrosphingosine. Fraction II usually contains less than 20 per cent of dihydrosphingosine, and some samples may contain very little. However, for preparative pur-

poses it is desirable to eliminate as much of the dihydro compound as possible by extraction of Fraction II with 100 volumes of methanol at room temperature for several hours. The insoluble material (largely dihydro-sphingosine sulfate) is removed and the sphingosine sulfate fraction is recovered from the filtrate. This material generally yields good quality triacetylsphingosine.

*A Acetylation of Sphingosine in Aqueous Alkali*—Crude sphingosine sulfate (Fraction B, II, b, 6.9 gm, 0.01 mole) was shaken with a mixture of 80 ml of 0.5 N sodium hydroxide and 100 ml of ether. A heavy emulsion formed as the free base was liberated and dissolved in the ether. Acetic anhydride (4.1 gm, 0.04 mole) and sodium hydroxide (40 ml of 2.5 N) were added and the mixture was vigorously shaken (with cooling under the tap) until the odor of acetic anhydride disappeared. The emulsion usually cleared during the acetylation and, if not, was broken up by the addition of methanol. The ether layer was separated, washed with water, and dried. The solvent was removed, leaving 5.0 gm of a gummy residue. Attempts to obtain crystalline N-acetylsphingosine from this material have, as yet, been unsuccessful.

*B Acetylation of N-Acetylsphingosine in Pyridine*—5 gm of crude N-acetylsphingosine were added to a mixture of 10 ml of pyridine and 10 ml of acetic anhydride. The solution warmed spontaneously and the solid dissolved. On standing at room temperature, the crystalline triacetyl derivative separated. After 2 hours the mixture was cooled in an ice bath and the precipitate was filtered, washed with 15 ml of cold acetone, and air-dried, giving 5.7 gm of triacetylsphingosine. This material was recrystallized from 30 ml of acetone, giving 4.9 gm of triacetylsphingosine (m p 99–101°,  $[\alpha]_D^{25} = -10.0^\circ$  (0.11 gm in 10 ml of chloroform)). Further recrystallization of this material from acetone yielded pure triacetylsphingosine.

A sample of mixed brain sulfates (1.8 gm of Fraction F, I and 6.0 gm of Fraction F, II) by the above process yielded 7.5 gm of crude N-acetylsphingosine and 6.0 gm of triacetylsphingosine (m p 99–101°,  $[\alpha]_D^{25} = -9.1^\circ$  (0.1 gm in 10 ml of chloroform)).

Fraction G, III (4.0 gm) gave 1.8 gm of impure triacetylsphingosine (m p 95–98°,  $[\alpha]_D^{27} = -7.9^\circ$  (0.1 gm in 10 ml of chloroform)).

Saponification equivalents were determined on several preparations of triacetylsphingosine as follows. The sample (0.5 mm) was dissolved by warming in 20 ml of 0.1 N potassium hydroxide in 90 per cent methanol. The solution was allowed to stand overnight at room temperature and the excess alkali was titrated back with 0.1 N acid. The results were uniformly in good agreement for the hydrolysis of the two O-acetyl groups. (0.2015 gm, 0.474 mm of triacetylsphingosine required 9.62 ml of 0.1 N alkali,

0.1624 gm, 0.382 mm required 7.50 ml of 0.1 N alkali) N-Acetylsphingosine was obtained as an amorphous solid by diluting the reaction mixtures with water.

#### IV Preparation of Triacetyldihydrosphingosine

*A Reduction of N-Acetylsphingosine*—Since dihydrosphingosine was desired for degradation studies, the reduction of various sphingosine derivatives was investigated. For reasons which will be discussed in a subsequent paper the reduction of triacetylsphingosine did not yield the desired dihydro derivative. However, N-acetylsphingosine was readily reduced with platinum in the Adams apparatus. Crude N-acetylsphingosine (4.3 gm) in 120 ml of 95 per cent ethanol was hydrogenated with 0.3 gm of platinum oxide. The uptake of hydrogen was 90 per cent of the theoretical. At the end of the reduction the precipitated N-acetyldihydrosphingosine was redissolved by heating and the catalyst was filtered. The filtrate was cooled in an ice bath, giving 3.8 gm of N-acetyldihydrosphingosine, melting at 120–123°. Recrystallization from 50 volumes of methanol yielded the pure compound (m.p. 125–126°,  $[\alpha]_D^{25} = +11.0^\circ$  (0.1 gm in 10 ml of chloroform-methanol, 1:1)).

C <sub>26</sub> H <sub>44</sub> NO <sub>2</sub>	Calculated	C 69.93, H 12.03, N 4.07
(313.5)	Found	" 70.23 " 12.30, " 3.87

*B Acetylation of N-Acetyldihydrosphingosine in Pyridine*—Several modifications of the acetylation procedure described for sphingosine (Section III, B) were necessary. N-Acetyldihydrosphingosine is much less soluble in pyridine than is N-acetylsphingosine. Hence the volume of the pyridine-acetic anhydride reaction mixture was doubled and the reaction mixture was warmed on the steam cone until a homogeneous solution was obtained. On standing at room temperature the solution solidified to a gel which was difficult to filter and retained acetone tenaciously. Therefore the reaction mixture (after 2 hours standing at room temperature) was cooled in an ice bath and treated with sufficient methanol to react with excess acetic anhydride. After 5 minutes the solution was transferred with ether to a separatory funnel containing iced dilute hydrochloric acid (1.0 ml of concentrated acid per ml of pyridine). The ether layer, containing the triacetyl derivative, was washed with dilute acid to remove the last traces of pyridine, with sodium bicarbonate solution to remove acetic and hydrochloric acids, and finally with water. The solution was dried and the ether removed, leaving the crude triacetyl derivative. This method is recommended for any fraction containing a high proportion of triacetyldihydrosphingosine. By the above procedure 5.0 gm of N-acetyldihydrosphingosine yielded 6.0 gm of crude triacetyldihydrosphingosine. This material

was recrystallized from 100 ml of ethanol, yielding 5.5 gm of pure crystalline material (m p 102–103°,  $[\alpha]_D^{30} = +18.0^\circ$  (0.1 gm in 10 ml of chloroform))

$C_{41}H_{83}NO_5$	Calculated	C 67.41, H 10.61, N 3.28
(427.6)	Found	" 67.25, " 10.60, " 3.41

The saponification equivalent of triacetyldihydrosphingosine (determined by the method used for triacetylsphingosine) gave a value agreeing with the hydrolysis of the two O-acetyl groups. N-Acetyldihydrosphingosine separated from the saponification mixture in crystalline form during the course of the reaction.

*C. Acetylation of Fraction I Sulfates*—1 Fraction G, I (5 gm) was acetylated in aqueous alkali in the usual way. Considerable difficulty was encountered, due to the separation of ether-insoluble material. This was finally brought into solution by the addition of methanol. The ether-methanol layer was separated and concentrated *in vacuo*. The residue was dissolved in 80 ml of hot methanol. The solution was cooled in an ice bath, yielding 2.5 gm of crystalline material (m p 110–114°). Recrystallization of this material from methanol gave 2.3 gm of N-acetyldihydrosphingosine (m p 122–124°). The methanolic mother liquors were concentrated and diluted with water, giving 2.0 gm of a waxy amorphous solid. This material gave 2.2 gm of triacetylsphingosine (m p 99–101°,  $[\alpha]_D^{27} = -10.0^\circ$  (0.1 gm in 10 ml of chloroform)) on further acetylation in pyridine.

2 The purified sulfate (1.3 gm) obtained by recrystallizing Fraction B, I from glacial acetic acid (see Section II) was converted to the free base, and the latter was treated with a mixture of 8 ml of pyridine and 6 ml of acetic anhydride. The solution turned to a stiff gel immediately. The triacetyl derivative was extracted with ether, after the reaction mixture was treated with methanol and iced hydrochloric acid. The crude derivative was recrystallized from 40 ml of ethanol, giving 1.2 gm of triacetyldihydrosphingosine (m p 100–102°,  $[\alpha]_D^{26} = +17.4^\circ$  (0.1 gm in 10 ml of chloroform)). From the filtrate was obtained 0.4 gm of material melting at 94–98° and giving a specific rotation of +3.3 (0.1 gm in 10 ml of chloroform).

3 Fraction A, I (5.0 gm) gave 3.7 gm of triacetyl derivative (m p 98–100°,  $[\alpha]_D^{26} = +4.5^\circ$  (0.1 gm in 10 ml of chloroform)). Fraction B, I (2.0 gm) gave 1.3 gm of triacetyl derivative (m p 96–99°,  $[\alpha]_D^{28} = +3.1^\circ$  (0.1 gm in 10 ml of chloroform)). Fraction A, I was obtained from crude spinal cord sphingolipide and Fraction B, I was prepared from the same material after recrystallization from glacial acetic acid. The purified material contained 90 to 95 per cent cerebroside. Since the two triacetyl

derivatives contained about the same proportion of triacetyldihydrosphingosine, these data indicate that dihydrosphingosine is an integral part of the cerebroside fraction.

#### V Preparation of Tribenzoylsphingosine

1 gm. of triacetylsphingosine was hydrolyzed by treatment with 20 ml. of 0.5 N methanolic potassium hydroxide for 8 hours on the steam bath under reflux. The cooled solution was diluted with 100 ml. of water and the base was extracted with ether. The ether solution was washed with water and dried. The ether was removed, giving 0.5 gm. of crystalline base melting at 79–81°. 290 mg. of this material were dissolved in 5 ml. of pyridine and 1.0 gm. of benzoyl chloride was added. After 3 hours at room temperature the reaction mixture was cooled in an ice bath and 1 ml. of water was added to hydrolyze excess benzoyl chloride. After 5 minutes the solution was diluted with 10 ml. of water and the precipitate was filtered and washed with water, giving 0.41 gm. of crude material melting at 110–117°. Recrystallization of this material from 20 ml. of ethanol gave 0.24 gm. of product melting at 118–121°. A second recrystallization from 10 ml. of ethanol yielded 0.15 gm. of material melting at 121.5–123.5° and giving a specific rotation of  $[\alpha]_D^{27} = -11.2^\circ$  (0.1 gm. in 10 ml. of chloroform).

$C_{33}H_{51}NO_3$	Calculated	C 76.56, H 8.07, N 2.29
(611 S)	Found	" 76.80, " 8.30, " 2.42

#### VI Preparation of Tribenzoyldihydrosphingosine

Crude sphingosine sulfate (Fraction E, II, 14 gm.) was suspended in 250 ml. of 1 N sodium hydroxide, and 100 ml. of ether were added. The mixture was shaken vigorously until the sulfate disappeared. As the free base dissolved in the ether a heavy emulsion formed. 9 gm. of benzoyl chloride were added in 3 portions with vigorous shaking and cooling under the tap. The emulsion gradually cleared and at the end of the reaction (with no odor of benzoyl chloride) the ether layer was separated, washed with water, and dried. The ether was removed, leaving 20 gm. of a white waxy residue. This was dissolved in 120 ml. of hot methanol and the solution was cooled overnight in the ice box. The benzoyl derivative separated in the form of a gel which was difficult to free of solvent. This behavior can be avoided partially by adding water to the hot methanol solution, causing the derivative to separate as an amorphous solid which is easily filtered and dried. The dried product weighed 12 gm. and liquefied at 70–90°. This material could not be obtained in a crystalline form from methanol, ethanol, benzene, or acetic acid.

The crude benzoyl derivative (8.0 gm.) was dissolved in 100 ml. of warm ethanol and reduced catalytically in the Adams apparatus with 0.2 gm. of

platinum oxide catalyst. The reduction proceeded rapidly (15 minutes) and N-benzoyldihydrosphingosine separated toward the end. The reduction mixture was warmed to redissolve the precipitate and the platinum was removed. The filtrate on cooling (or preferably on the addition of water) deposited 7.5 gm of amorphous N-benzoyldihydrosphingosine liquefying at 114–118°. The amorphous benzoyl derivative (7.5 gm) was suspended in 75 ml of pyridine and 15 ml of benzoyl chloride were slowly added from a dropping funnel (reaction mixture protected from moisture) with cooling and shaking. The mixture was allowed to stand at room temperature for 3 hours. Cold water (50 ml) was slowly added. The precipitate originally present (pyridine hydrochloride) disappeared and a new crystalline solid separated. This was filtered, washed thoroughly with water, and recrystallized from 600 ml of hot ethanol, yielding 5.0 gm of beautifully crystalline tribenzoyldihydrosphingosine melting at 140–142°. Two additional recrystallizations raised the melting point to 144–145° with little loss of material. This substance gave a specific rotation of  $[\alpha]_D^{27} = -31^\circ$  (0.1 gm in 10 ml of chloroform) or  $-19^\circ$  (0.1 gm in 10 ml of pyridine), and gave the correct analytical data for tribenzoyldihydrosphingosine.

$C_{33}H_{51}NO_5$	Calculated	C 76.31, H 8.38, N 2.28
(613.8)	Found	" 76.42, " 8.54, " 2.30

### VII N-Benzoyldihydrosphingosine

Tribenzoyldihydrosphingosine (6.0 gm) was dissolved in 1200 ml of hot ethanol (95 per cent) and a solution of 1.8 gm of potassium hydroxide in 100 ml of ethanol was added. The mixture was allowed to stand 12 hours at room temperature and was neutralized to phenolphthalein with hydrochloric acid, and concentrated *in vacuo*. Water was added and the gelatinous precipitate which formed was filtered, washed thoroughly with water, and dried, giving 3.50 gm (88 per cent) of an amorphous solid liquefying at 116–119°. N-Benzoyldihydrosphingosine is less soluble in ethanol and in ether than is N-benzoylsphingosine. It has not been obtained in a crystalline state from alcohol, benzene, or acetic acid.

3 gm of tribenzoyldihydrosphingosine were refluxed for 7 hours with 100 ml of methanolic-sulfuric acid (10 per cent by weight). The product consisted mainly of the N-benzoyl derivative (even very prolonged refluxing of the tribenzoyl derivative with methanolic-sulfuric acid does not remove completely the N-benzoyl group). The crude N-benzoyl derivative was heated under a reflux for 5 hours in 100 ml of 5 per cent methanolic-potassium hydroxide. The reaction mixture was cooled and diluted with water, giving 1.5 gm of crude base (m.p. 83–90°).

### VIII Benzoylation of Crude Sulfate Fractions

It has not been possible to obtain pure tribenzoylsphingosine or triben-



zoyldihydrosphingosine from the crude sulfate fractions. Fraction I gave a product containing mainly tribenzoyldihydrosphingosine but the pure derivative has been obtained only after catalytic reduction of these products or of the crude N-benzoylsphingosine from Fraction II. Fraction II gave tribenzoyl derivatives melting over a wide range. Certain of these appear to contain a considerable amount of the dihydro derivative on the basis of melting point and optical rotation. Others melt in the general range for tribenzoylsphingosine (118–121°), but our failure to obtain that compound in a pure state from sphingosine makes it difficult to evaluate the purity of such products. The following experiments with Fractions I and II are typical.

**A. Benzoylation of Fraction II**—Crude sphingosine sulfate (Fraction G, II, 25 gm.) was shaken with 120 ml. of 1.0 N sodium hydroxide and 200 ml. of ether. The addition of 25 ml. of methanol hastened the disappearance of the sulfate. As the free base was liberated and dissolved in the ether layer, a heavy emulsion formed. To this mixture were added, in 3 portions, 21 gm. (2 moles) of benzoyl chloride and 220 ml. of 2 N sodium hydroxide. The reaction mixture was shaken vigorously and cooled under the tap until the odor of benzoyl chloride no longer could be detected. The ether layer was separated, washed with water, dried, and the ether removed, leaving 25 gm. of a waxy solid, liquefying at 60–80°.

Half of this material (12.5 gm.) was benzoylated further in the usual way with 125 ml. of pyridine and 25 gm. of benzoyl chloride. The product was a sticky solid (11 gm.) which, after two recrystallizations from 350 ml. of ethanol, weighed 10.5 gm. and melted at 110–123°,  $[\alpha]_D^{30} = -7.8^\circ$  (0.1 gm. in 10 ml. of chloroform).

The other half of the crude N-benzoylsphingosine was reduced catalytically in 150 ml. of glacial acetic acid. (This solvent is somewhat preferable to ethanol, since the N-benzoyl derivatives are more soluble in it.) The platinum catalyst was removed and water was added to the filtrate, precipitating the crude N-benzoyldihydrosphingosine. The precipitate was removed and dried *in vacuo*. It was benzoylated in 150 ml. of pyridine with 25 gm. of benzoyl chloride. The crude product weighed 12.0 gm. and melted at 100–120°. It was recrystallized from 800 ml. of ethanol, giving 8.8 gm. of nearly pure tribenzoyldihydrosphingosine melting at 140–143°.

**B. Benzoylation of Fraction I**—Fraction G, I (5.0 gm.) was treated with 80 ml. of 1.0 N sodium hydroxide, 50 ml. of ether, 20 ml. of methanol, and 3.0 gm. of benzoyl chloride. The sulfate dissolved very slowly and the N-benzoyl derivative separated partially from the ether.<sup>7</sup> The crude benzoyl derivative weighed 5.5 gm. and melted at 95–100°. This material on

<sup>7</sup> Since the dihydro derivative is not very soluble in ether, it is preferable to benzoylate fractions high in dihydrosphingosine by converting the sulfate to the free base in alcoholic alkali and benzoylating the base directly in pyridine.

further benzylation in pyridine gave 6.6 gm of tribenzoyl derivative melting at 110–130°. Recrystallization of this product from 700 ml of hot ethanol yielded 4.2 gm of nicely crystalline material melting at 133–137°. The specific rotation of this material ( $[\alpha]_D^{25} = -28.4^\circ$  (0.1 gm in 10 ml of chloroform)) indicated it to contain mainly tribenzoyldihydrosphingosine. These results are typical of many similar experiments.

### IX Reduction of Tribenzoylsphingosine

Attempts to reduce tribenzoylsphingosine directly to the dihydro derivative in glacial acetic acid were not successful. The hydrogen uptake con-

TABLE II  
*Iodine Numbers of Sphingosine, Dihydrosphingosine, and Their Derivatives*

Compound	Iodine No		Optical rotation (chloroform)
	Theoretical	Found	
Triacetylsphingosine	59.7	52.7	-11.7
Triacetyldihydrosphingosine	0	3.2	+18.0
Mixtures of 2 compounds above		30.5	+3.0
		39.4	-3.3
		37.9	-3.5
		42.8	-5.8
Tribenzoylsphingosine	41.5	35.3	-5.3
Tribenzoyldihydrosphingosine	0	4.8	-29.5
Crude sphingosine sulfate (Fraction D, II, b)	72.9	54.0	
Dihydrosphingosine sulfate*	0	12.1	

\* Fraction I sulfate recrystallized from glacial acetic acid

siderably exceeded the theoretical and the products melted over a wide range. Thus 6.0 gm of tribenzoylsphingosine (m.p. 118–122°) reduced with 0.3 gm of platinum oxide in 150 ml of warm glacial acetic acid absorbed over 2 moles of hydrogen (pressure drop 1.85 pounds, theoretical 0.85 pound). The solution was warmed and the catalyst removed. The filtrate on cooling deposited 3.8 gm of crystalline material (m.p. 125–139°). Recrystallization from ethanol gave 3.7 gm (m.p. 137–141°). The acetic acid filtrate on dilution with water gave 1.5 gm (m.p. 100–115°).

It seems probable that partial reduction of the benzene ring may have occurred, as well as another side reaction, which will be discussed in a subsequent paper. However, it is possible to reduce N-benzoylsphingosine in good yields to N-benzoyldihydrosphingosine, as described in Sections VI and VIII, A.

## X Iodine Number Determinations

Iodine numbers were determined on a variety of samples of sphingosine and dihydrosphingosine and their derivatives, by the method of Hanus (15) with mercuric acetate as a catalyst (16) and the method of Rosenmund and Kuhlmann (17). Salts of the bases gave erratic results, especially with the Hanus method. Acyl derivatives gave reproducible figures, but the results were always less than the theoretical value. Certain of the data are summarized in Table II. These results were obtained with the Rosenmund-Kuhlmann procedure.

## SUMMARY

Dihydrosphingosine has been characterized as a component of the sphingolipides of beef brain and spinal cord. It is present in a higher concentration in spinal cord than in brain. This is the first observation of the occurrence of dihydrosphingosine in nerve tissue of higher animals, although Lesuk and Anderson (13) isolated it from *Cysticercus fasciolaris*.

Methods have been described for preparing sphingosine, dihydrosphingosine and their N-acetyl, N-benzoyl, triacetyl, and tribenzoyl derivatives.

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# BIOCHEMISTRY OF THE SPHINGOLIPIDES\*

## III STRUCTURE OF SPHINGOSINE

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(Received for publication, May 5, 1947)

Sphingosine was discovered by Thudichum (2, 3), who isolated it from the hydrolysis products of phoscosin, characterized it as a base of empirical composition  $C_{17}H_{35}NO$ , and described a number of its salts. Worne and Thierfelder (4) reported that sphingosine absorbed bromine, indicating unsaturation in the molecule. Several years later, Levene and Jacobs (5) in a more thorough study characterized sphingosine as an unsaturated monoimmodihydroxy alcohol. They prepared a triacetyl derivative (m.p. 102–103°) and reduced sphingosine to dihydrosphingosine with palladium as the catalyst. In the same year Thomas and Thierfelder (6) also reported the preparation of triacetylsphingosine (m.p. 100–102°).

Lapworth (7) and Levene and West (8, 9) oxidized sphingosine with chromic acid and obtained an acid which was identified as *n*-tridecanoic acid, although its melting point did not agree too well with that of an authentic sample. Levene and West also oxidized dihydrosphingosine in a similar fashion and obtained an acid melting at 60–61°, which they identified as *n*-pentadecanoic acid.

These results indicate that sphingosine has a  $C_{17}$  straight chain with a double bond between carbon atoms 4 and 5, and with two hydroxyl groups and an amino group on the first 3 carbon atoms. Levene and West (9) also investigated the ozonolysis of sphingosine. The nitrogen-containing fragment was oxidized further with nitric acid to a substance tentatively identi-

\* The authors wish to express their deep appreciation to The Upjohn Company for a grant in support of this work.

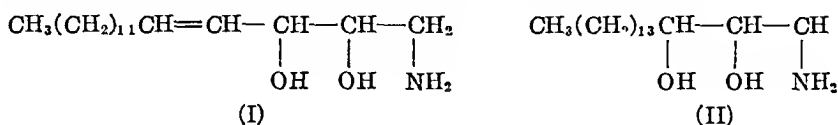
The material presented in this paper was taken from the theses submitted by F. J. Glick, W. P. Norris, and G. E. Phillips to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry. A preliminary report of a portion of this work has been published previously (1).

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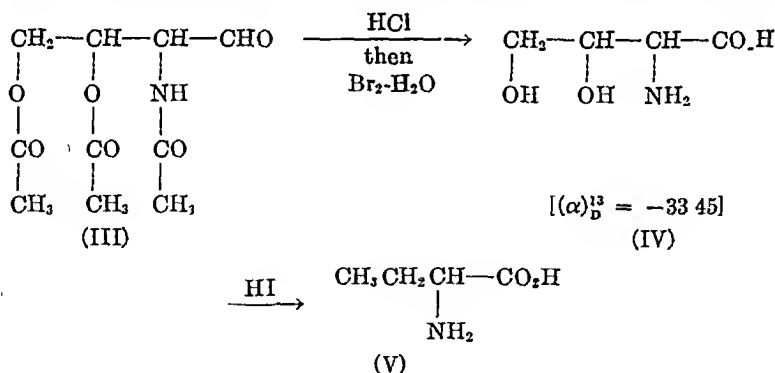
§ Present address, The Maltine Company, New York.

fied as *meso*-tartaric acid. On the basis of these data the formulas for sphingosine (I) and dihydrosphingosine (II) were proposed



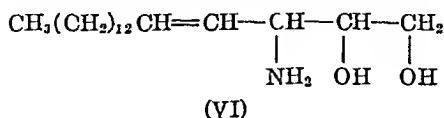
Doubt concerning the occurrence of an odd numbered carbon chain in a natural product led Klenk and coworkers (10, 11) to reinvestigate the structure of sphingosine. They repeated the chromic acid oxidation of sphingosine and dihydrosphingosine and found that the products were myristic and palmitic acids, respectively, rather than tri- and pentadecanoic acids, as previously reported. Furthermore, ozonolysis of triacetylsphingosine yielded myristaldehyde and myristic acid. These data require a  $\text{C}_{18}$  structure for sphingosine, and in confirmation of this view Klenk found that the analytical data for triacetylsphingosine and for salts of dihydrosphingosine (10), as well as many of the analyses of previous workers (10, 12), fitted the  $\text{C}_{18}$  structure better than the  $\text{C}_{17}$ .

Klenk and Diebold (11) isolated the nitrogenous fragment from the ozonolysis of triacetylsphingosine and identified it as III by the reactions



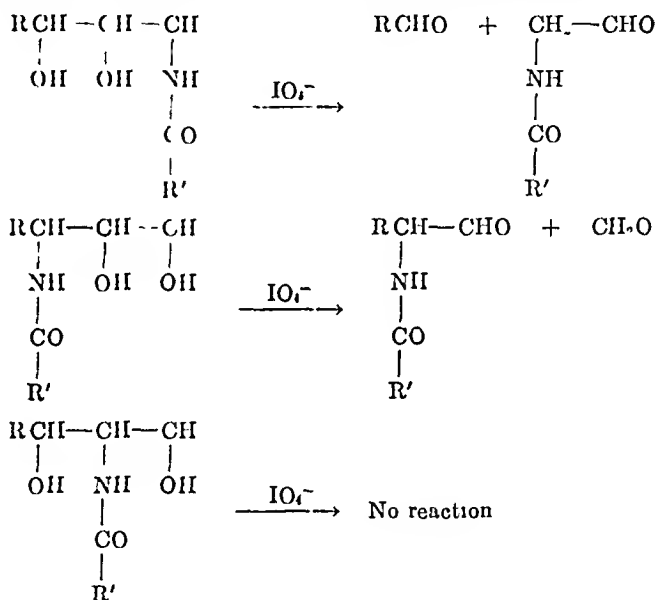
shown in III to V. Compound IV was characterized by analyses, melting point, and optical rotation. Compound V was identified as DL- $\alpha$ -amino-*n*-butyric acid by analyses and melting point.

On the basis of these observations Klenk and Diebold (11) proposed



structure VI for sphingosine. Although the evidence for VI seemed good, an absolute identification had not been made of either IV or V. It seemed

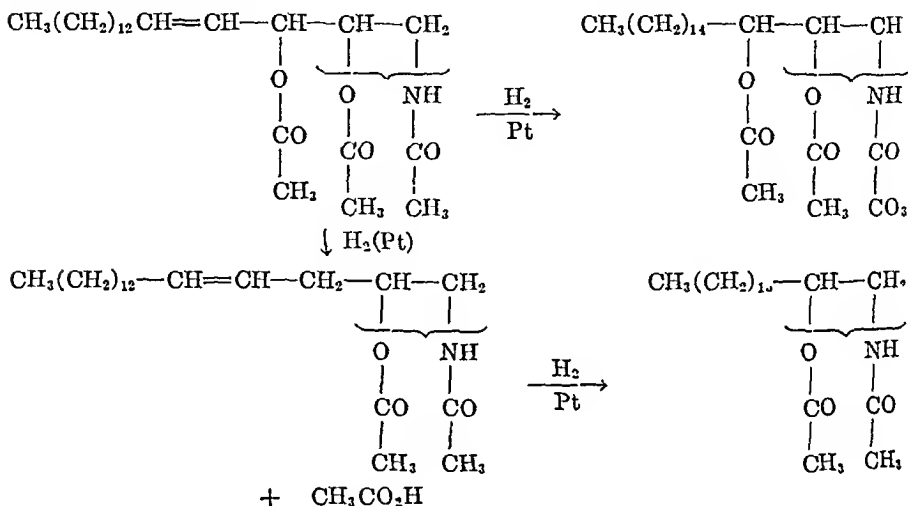
desirable, therefore, to confirm Klenk's results, either by synthesis of the various isomers of IV or by independent degradative studies on sphingosine.<sup>1</sup> The latter alternative seemed more attractive, since the results thus obtained would be informative, regardless of the correctness of Klenk's conclusions. Furthermore sphingosine afforded a suitable compound for degradation studies with periodic acid, a reagent which oxidizes 1,2-glycols (14, 15) and 1,2-amino alcohols (15, 16) but does not attack an N-acyl-1,2-amino alcohol (15, 16). It seemed probable, therefore, that the three possible structures for dihydrosphingosine would be readily distinguished by this reagent, as shown in the accompanying equations.



In order to avoid possible interference by the double bond in sphingosine,<sup>1</sup> the degradation studies were performed on dihydrosphingosine derivatives. In the course of preparing these substances a study was made of the catalytic reduction of triacetylsphingosine. To our surprise anomalous results were obtained. The hydrogen uptake by triacetylsphingosine amounted to 1.5 to 1.8 moles per mole instead of the expected 1 mole. Furthermore 0.5 to 0.7 mole of acid was produced in the reduction. It seemed evident, therefore, that hydrogenolysis of an acetoxyl group had occurred. Such a reaction is characteristic of an allylic system (17) but not of an unactivated

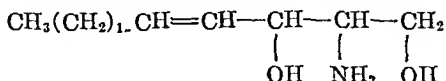
<sup>1</sup> Simultaneously with the appearance of our original note (1), Niemann and Nichols (13) described the preparation of two diastereoisomeric  $\alpha$ -amino  $\beta, \gamma$ -dihydroxy-*n*-butyric acids and found that neither of them gave the rotation reported by Klenk and Diebold for IV.

acetoxy group This result strongly indicates that one of the acetoxy groups of triacetylsphingosine is adjacent to a double bond, *i.e.*, in an allylic position The fact that triacetyldihydrosphingosine is stable to catalytic reduction strengthens such a view The hydrogenation of triacetylsphingosine would thus be represented as in the accompanying formulas, involving a competition in the first step between hydrogenolysis and hydrogenation



This result was the first evidence against the correctness of the sphingosine structure proposed by Klenk

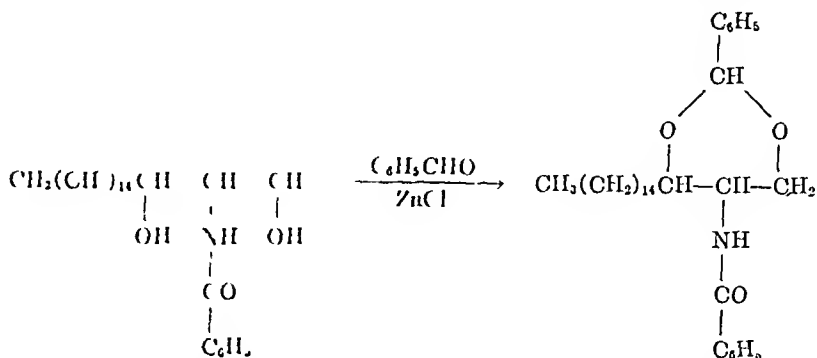
As indicated in a previous paper (18), it is possible to reduce N-acyl derivatives of sphingosine to the dihydro compounds Both N-acetyl and N-benzoyldihydrosphingosine were prepared in this way and subjected to treatment with periodic acid under a variety of conditions In no case was any oxidation detected by quantitative determination of periodate In each case an excellent yield of the unchanged derivative was recovered These results therefore establish the absence of a 1,2-glycol group in N-acylsphingosines Sphingosine therefore has neither of the previously suggested formulas but it is best represented as follows



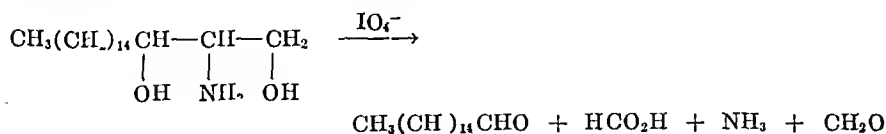
The above argument is valid only if the three functional groups of sphingosine are on the terminal 3 carbon atoms, as postulated by Levene and Klenk In this respect further studies fully substantiated the conclusions of the earlier workers N-Benzoyldihydrosphingosine gave a nicely crystal



line benzylidene derivative on treatment with benzaldehyde and zinc chloride. Since this reaction is characteristic only of 1,2- and 1,3-glycols, and since the former is eliminated by the periodate results, the two hydroxyl



groups in N-benzoyldihydrosphingosine must be in the 1,3 position. Additional information was furnished by a quantitative study of the oxidation of dihydrosphingosine by sodium periodate. This reaction was complicated by the precipitation of an imine salt when sodium periodate or periodic acid was added to an alcoholic or ethereal solution of dihydrosphingosine. Different experimental conditions were tested in an attempt to avoid this difficulty. It was discovered that the reaction remained homogeneous in a dioxan-methanol mixture and this solvent was used for analytical studies. However, for isolation of the products it was found more satisfactory to treat an ethereal solution of the base with aqueous sodium periodate. Dihydrosphingosine consumes about 2 moles of periodate and yields formic acid, formaldehyde, ammonia, and palmitaldehyde, the first three in equivalent amounts. The yield of palmitaldehyde was not estimated, due to losses in converting it into derivatives. The semicarbazone and the thiosemicarbazone were prepared. Analytical data and melting points of these compounds agreed well with those for palmitaldehyde. These data show by an independent method that sphingosine has a  $\text{C}_{18}$  chain. They also require that the three functional groups in sphingosine be located on adjacent carbon atoms and that one of these be a terminal carbon, thus establishing conclusively the structure of sphingosine.



This structure of sphingosine suggests an  $\alpha$ -amino- $\beta$ -hydroxy acid as a possible bioprecursor, and also opens up a possibility of synthesizing dihy-

drospingosine by catalytic reduction of an ester of  $\alpha$ -amino- $\beta$ -hydroxy-stearic acid. These problems are being studied in our laboratories at the present time.

#### EXPERIMENTAL

*Reduction of Triacetylspingosine*—Triacetylspingosine (6.18 gm, 14.5 mm) was dissolved in 130 ml of ethanol (with warming) and reduced in the Adams apparatus with 200 mg of freshly prepared platinum oxide<sup>2</sup> (19). The uptake of hydrogen was 23.1 mm. In a control run 200 mg of platinum oxide required 1.6 mm of hydrogen. The derivative therefore absorbed 21.5 mm or 150 per cent of the theory. The crude product (5.7 gm) appeared to be only partially crystalline and melted at 87–97°. On repeated recrystallization from acetone 1.3 gm of material melting at 100–103° were obtained. The residues were combined and recrystallized once from acetone, giving 4.0 gm of product melting at 87–90°. This material gave the following analyses:

$C_{27}H_{45}NO_3$ (369.57) <sup>3</sup>	Calculated	C 71.49, H 11.73, N 3.79
$C_{27}H_{45}NO_3$ (427.61) <sup>4</sup>	"	" 67.41, " 10.61 " 3.28
	Found	" 68.66, " 10.81 " 3.66

An O-acetyl determination on this material (as described in a previous paper (18)) showed only 1.3 moles per mole instead of the theoretical value of 2.0 for triacetyldihydrospingosine.

Triacetylspingosine (5.26 gm, 12.4 mm) was reduced as described above. The corrected uptake of hydrogen was 21.0 mm or 170 per cent of the theory. The solution on titration with sodium hydroxide required 8.0 mm of alkali. This is equivalent to the hydrogenolysis of 65 per cent of one acetoxy group.

A number of experiments were carried out in which temperature and ratio of catalyst to triacetyl derivative were varied, but the results were substantially the same. Further details on the nature of the reduction product will be reported in a subsequent paper.

*Reaction of N-Acetyldihydrospingosines with Periodic Acid*—N-Benzoyldihydrospingosine (4.3 gm, m.p. 116–118°) was dissolved in 40 volumes of methanol and added to a solution of 8 gm of periodic acid in 200 ml of methanol. The solution was allowed to stand for 24 hours at room temperature and then was concentrated under reduced pressure to 150 ml. Warm water was added, precipitating 4.0 gm of unchanged N-benzoyldihydro-

<sup>2</sup> Commercial platinum black gave considerably less hydrogenolysis than did platinum formed *in situ* by reduction of the oxide.

<sup>3</sup> Diacetyl derivative of aminohydroxyoctadecane.

<sup>4</sup> Triacetyldihydrospingosine.

sphingosine. Since this substance is in amorphous powder, it was converted to tribenzoyldihydrosphingosine for characterization. An 85 per cent yield of product melting at 113–115° was obtained.

Similar results were obtained if the reaction mixture was allowed to stand at 50° for 24 hours. The use of dioxan as the solvent also gave similar results. Attempts to oxidize N-benzoyldihydrosphingosine with sodium metaperiodate were complicated by the insolubility of the reagent in methanol and the slight solubility of the derivative in aqueous methanol. However, an experiment was carried out in which 4.0 gm. of N-benzoyldihydrosphingosine were dissolved in 300 ml. of methanol and a solution of 5 gm. of sodium periodate in 50 ml. of water was added. The precipitate was suspended by stirring for 72 hours. The reaction mixture was diluted with 1 liter of warm water and the precipitate was filtered, washed thoroughly with water, and dried, giving 3.6 gm. of unchanged N-benzoyldihydrosphingosine.

N-Acetyldihydrosphingosine (1.0 gm., m.p. 122–123°) was dissolved in 50 ml. of methanol. 10 ml. of a 1 per cent solution of periodic acid in methanol were added and the solution was made up to 100 ml. with methanol. Aliquot portions of 2 ml. each were removed after 1, 3, 5, and 24 hours. The sample was added to 25 ml. of water. Potassium iodide was added and the solution was acidified with sulfuric acid and titrated with sodium thiosulfate. There was no difference between the values thus obtained and those of a control solution of the same composition, except for the N-acetyldihydrosphingosine. The remainder of the reaction mixture was concentrated *in vacuo*, water was added, and the precipitate was removed and recrystallized from methanol. The product weighed 0.5 gm. and melted at 123–124°. Acetylation of this material in pyridine gave triacetyldihydrosphingosine in 80 per cent yield.

*Benzal Derivative of N-Benzoyldihydrosphingosine*—The N-benzoyldihydrosphingosine (1.5 gm.) from a periodic acid reaction was shaken overnight with 10 ml. of benzaldehyde and 3 gm. of anhydrous zinc chloride. The mixture became viscous and a solid separated. The reaction mixture was taken up in 200 ml. of ether. The solution was shaken for 1 hour with 20 gm. of sodium carbonate and decanted into 500 ml. of 4 per cent aqueous sodium carbonate. The emulsion which formed was allowed to separate and the ether layer was removed and dried. Evaporation of the ether left 1.5 gm. of crystalline material melting at 140–145°. This material was recrystallized from 250 ml. of ethanol, giving 1.3 gm. of product (m.p. 142–147°). A second recrystallization from 200 ml. of ethanol yielded 0.4 gm. (m.p. 142–148°, specific rotation +37.4° (0.1 gm. in 10 ml. of chloroform)).

C <sub>21</sub> H <sub>47</sub> NO <sub>2</sub> (493.7)	Calculated	C 77.85, H 9.58, N 2.84
	Found	" 78.00 " 9.70 " 2.98

An additional 0.5 gm of crystalline material melting at 138–145° was obtained from the mother liquors

Found, C 78.03, H 9.72, N 2.92

Apparently this material represents a mixture of the two benzal derivatives possible as the result of the introduction of a new asymmetric carbon atom into the molecule

*Oxidation of Dihydrosphingosine with Periodate Analytical Experiment*—To a solution of dihydrosphingosine (52.7 mg, 0.175 mm) in 10 ml of dioxan and 2 ml of methanol were added 5.0 ml of a 5 per cent methanolic solution of periodic acid. 3 hours later there was a decrease of 7.4 ml of 0.1 N sodium thiosulfate in the titration value for the reaction mixture, as compared to the control solution. This corresponds to 0.37 mm of periodate consumed or 2.1 moles of periodate per mole of dihydrosphingosine. In similar experiments the amount of periodate required varied from 2.06 to 2.48 moles per mole of dihydro base.

*Isolation of Oxidation Products*—In order to isolate the products of the dihydrosphingosine oxidation the reaction was carried out in an aqueous solution. Under these conditions a highly insoluble salt of dihydrosphingosine (iodate or periodate) was precipitated, even when the pH of the reaction mixture was raised by adding 10 per cent sodium carbonate solution. After considerable preliminary experimentation, the following conditions were adopted. The experiments described below are typical of several others.

A solution of 0.868 gm of dihydrosphingosine in 30 ml of ether was stirred with 35 ml of 10 per cent aqueous sodium periodate, for 5 hours at room temperature. The material precipitating from the ether layer was collected and dried in a sintered glass filter, giving 0.8 gm of a dihydrosphingosine salt. Calculated on the periodate salt, this amount of material is equivalent to 488 mg or 56 per cent of the original dihydrosphingosine.<sup>5</sup> The ether layer from the reaction mixture was washed once with water and concentrated *in vacuo*, giving a semisolid waxy residue. This material was dissolved in 4 ml of ethanol. Semicarbazide hydrochloride (0.3 gm) and sodium acetate (0.45 gm) were added and the solution was refluxed for several minutes and warmed for an additional 2 hours. On cooling, a crystalline semicarbazone separated. After several recrystallizations from dilute alcohol the material melted at 106.5–107°. Palmitaldehyde semicarbazone is reported to melt at 107° (20).

<sup>5</sup> The salt is extremely insoluble in water or hot alcohol. It was refluxed with aqueous alcoholic potassium hydroxide but did not dissolve completely. The solution was extracted with ether and the ether solution was evaporated. The residue gave tribenzoyldihydrosphingosine (m.p. 143–144°) on treatment with benzoyl chloride in pyridine. The aqueous alcoholic solution from the ether extraction

$C_{17}H_{33}N_3O$ (297.17) <sup>a</sup>	Calculated	C 68.63, H 11.86, N 14.16
$C_{16}H_{31}N_3O$ (283.15) <sup>7</sup>	"	" 67.79 " 11.73 " 14.85
	Found	" 68.68, " 11.68, " 14.33

The aqueous layer and washings were diluted to 50 ml, of which 6 ml were mixed with 11 ml of 10.33 per cent aqueous solution of dimedon (21). After 24 hours the precipitate was filtered, washed, and dried, giving 44 mg of the dimedon derivative of formaldehyde, melting at 189°. This yield is equivalent to 356 mg of dihydrosphingosine.

10 ml samples of the formaldehyde-free filtrate were mixed with concentrated sodium hydroxide and distilled into 10 ml of 0.01 N hydrochloric acid in a micro-Kjeldahl apparatus. The average consumption of hydrochloric acid was 3.01 ml. This is equivalent to 2.12 mg of nitrogen in the original 6 ml sample or 17.7 mg in the entire oxidation solution. This amount of nitrogen is equivalent to 380 mg of dihydrosphingosine.

A solution of 1.0 gm of dihydrosphingosine in 125 ml of ether was stirred while 20 ml of 0.3 N sodium periodate were added dropwise. This method of adding the periodate did not prevent the usual formation of a white solid precipitate. The yields of oxidation products were slightly lower than in other experiments, possibly because an excess of periodate was not used. The oxidation products were isolated as in the preceding experiment.

The fatty aldehyde was heated 1 hour under a reflux with a solution of 150 mg of thiosemicarbazide in 35 ml of absolute ethanol. The solution was allowed to stand overnight at room temperature and was then concentrated to 4 ml and cooled. The precipitate was filtered and washed with 2 ml of cold ethanol. Excess thiosemicarbazide was extracted with 50 ml water and the residue was recrystallized from ethanol, giving 244 mg of a thiosemicarbazone. After two additional recrystallizations from dilute ethanol the material melted at 106–107°. The thiosemicarbazone of palmitaldehyde is reported to melt at 109° (22).

$C_{17}H_{33}N_3S$ (313.55) <sup>8</sup>	Calculated	C 65.12, H 11.25, N 13.41
$C_{16}H_{31}N_3S$ (299.53) <sup>9</sup>	"	" 64.16, " 11.11, " 14.03
	Found	" 65.27, " 11.20, " 13.43

The aqueous layer and ether washings yielded 193 mg of the formaldehyde-dimedon derivative (m.p. 189°) equivalent to 200 mg of dihydrosphingosine. The formaldehyde-free filtrate was neutralized to pH 7.2 with barium hydroxide and concentrated to dryness *in vacuo*. The residue was dissolved in 50 ml of water, and 1.5 ml of 85 per cent phosphoric acid were

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liberated iodine when added to an acid solution of potassium iodide. These data indicate that the material is either the iodate or periodate salt of dihydrosphingosine.

<sup>a</sup> Semicarbazone of palmitaldehyde.

<sup>7</sup> Semicarbazone of pentadecanal.

<sup>8</sup> Thiosemicarbazone of palmitaldehyde.

<sup>9</sup> Thiosemicarbazone of pentadecanal.

added The solution was distilled *in vacuo* through an efficient condenser and the distillate was collected in a trap cooled in an ice-salt bath The formic acid was titrated with sodium hydroxide at 25° to a pH of 7.0 with a Beckman pH meter with traveling electrodes Exactly 7.0 ml of 0.1 N sodium hydroxide were required This is equivalent to 210 mg (0.7 mm) of dihydrosphingosine A quantitative permanganate oxidation on this solution showed it to contain 32 mg of formic acid (equivalent to 209 mg of dihydrosphingosine)

#### SUMMARY

Triacetylsphingosine undergoes partial hydrogenolysis of an acetoxy group on catalytic reduction, indicating that the structure proposed by Klenk and Diebold for sphingosine is not correct The failure of N-acylsphingosines to be oxidized by periodate and the oxidation of dihydrosphingosine to palmitaldehyde, formic acid, ammonia, and formaldehyde establish the structure of dihydrosphingosine as 1,3-dihydroxy-2-amino-octadecane These results in conjunction with the earlier experiments of Levene and of Klenk show that sphingosine is 1,3-dihydroxy-2-amino-octadecene-4

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# BIOCHEMISTRY OF THE SPHINGOLIPIDES\*

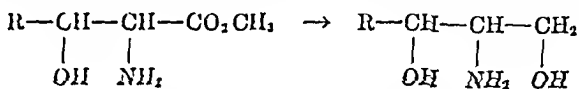
## IV SYNTHESIS OF COMPOUNDS RELATED TO SPHINGOSINE

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(Received for publication, May 9, 1947)

In a previous paper (1) evidence was presented establishing the structure of dihydrosphingosine as 1,3-dihydroxy-2-amino-octadecane. As a method of approaching the synthesis of compounds of this type, the reduction of the corresponding  $\alpha$ -amino- $\beta$ -hydroxy esters offered considerable promise



(Dihydrosphingosine,  $\text{R} = \text{C}_{15}\text{H}_{31}$ )

Levene and coworkers found that esters of  $\alpha$ -amino acids were reduced catalytically to amino alcohols over copper chromite (2) or Raney's nickel catalyst (3, 4), and that no racemization occurred when Raney's nickel was employed as the catalyst. With a large quantity of catalyst and hydrogen pressures of 2200 pounds the reduction proceeded rapidly at temperatures of 40-70° and excellent yields of product were obtained. It seemed probable that  $\alpha$ -amino- $\beta$ -hydroxy esters would behave similarly, with the possibility, however, that the  $\beta$ -hydroxyl group might be lost in the process. Therefore, as model compounds, the methyl esters of DL-allothreonine and DL-threonine were subjected to reduction with a large quantity of Raney's nickel as the catalyst. In each case the reduction proceeded rapidly and was complete before the temperature of the bomb reached 80°. The products were viscous oils which readily yielded crystalline derivatives. The tribenzoyl derivative from allothreonine (1,3-dibenzoyl-2-benzamido-*n*-butane) melted sharply at 155-156°, and there was little indication of the presence of a second isomer. Evidently little or no epimerization occurred.

\* The authors wish to express their deep appreciation to The Upjohn Company for a grant in support of this work. The material presented in this paper was taken from the theses submitted by W. P. Norris and Harriet E. Rockwell to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry.

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during the esterification-reduction process with allothreonine. The results were less clean-cut in the case of threonine. The tribenzoyl derivative, after repeated recrystallization from aqueous methanol and petroleum ether, softened at 127° and melted at 133–135°. Since this material gave the correct analytical data for the tribenzoyl derivative, it must have consisted of a mixture of the two possible racemic forms. It would appear that epimerization (extent undetermined) had occurred in the reduction or esterification of threonine. A further study of this point is being made. However, these results afford some hope of obtaining the four diastereoisomeric 1,3-dihydroxy-2-aminooctadecanes by reduction of the corresponding isomers of methyl  $\alpha$ -amino- $\beta$ -hydroxystearate. Such a conversion would not only provide a synthesis for dihydrosphingosine but would also furnish information as to the stereochemical configuration of the base. To this end the synthesis of the  $\alpha$ -amino- $\beta$ -hydroxystearic acids is being investigated.

As a further model compound methyl  $\alpha$ -aminostearate was subjected to reduction with Raney's nickel catalyst. The theoretical amount of hydrogen was absorbed rapidly and a 70 per cent yield of 1-hydroxy-2-aminooctadecane was obtained. This compound we believe to be *DL*-sphingine<sup>1</sup> on the basis of evidence which will be reported later. This smooth reduction of a long chain ester is encouraging as regards the synthesis of dihydrosphingosine.

Adkins and Reeve (6) reported the synthesis of a mixture of *DL*-threonine and *DL*-allothreonine by the reduction of ethyl oximinoacetoacetate (or its *O*-methyl ether). We found that with a large amount of catalyst the oximino ester was reduced readily in one step through the amino ester to 1,3-dihydroxy-2-amino-*n*-butane. The product on benzylation yielded mainly the derivative melting at 155–156°.

#### EXPERIMENTAL

*Preparation of Esters for Reduction*—Ethyl oximinoacetoacetate and the *O*-methyl ether were prepared according to directions of Adkins and Reeve (6). The methyl esters of *DL*-threonine and *DL*-allothreonine were obtained by the method of Fischer and Suzuki (7). The hydrochloride of *DL*-threonine methyl ester was obtained as a white crystalline solid melting at 112–115° by precipitation from an ethanol solution with ether. The yield was 88 per cent. The hydrochloride was converted to the free amino ester with an equivalent amount of sodium methylate. A 90 per cent yield of *DL*-threonine methyl ester melting at 35–36° was obtained. *DL*-Allothreonine methyl ester was obtained in the same manner. The hydrochloride

<sup>1</sup> Levene and West (5) first prepared sphingine by the reduction of dihydrosphingosine with hydriodic acid.



melted at 111–112°. The hydrochloride of methyl  $\alpha$ -aminostearate was prepared by the procedure of Fischer and Kropp (8) in 80 per cent yield. It was recrystallized from ethyl acetate (m p 109–110°). This hydrochloride is soluble in hot water but not in cold and is insoluble in ether. The free amino ester was recrystallized from aqueous methanol (m p 48–49°).

$C_{17}H_{33}NO_2$ (313.51)	Calculated	C 72.79, H 12.54, N 4.47
	Found	" 72.91, " 12.43, " 4.52

### Reduction of Esters

*Methyl Ester of DL-Allothreonine*—The ester (10 gm) was dissolved in 75 ml of anhydrous methanol, and 25 gm of Raney's nickel catalyst were added. The mixture was hydrogenated at 2300 pounds pressure of hydrogen as the temperature of the bomb was raised slowly. The reduction was completed below 80°. The product was a viscous basic oil which could not be induced to crystallize.

The crude base (2.5 gm) was dissolved in 50 ml of anhydrous pyridine, and 15 gm of benzoyl chloride were added slowly with cooling and shaking (protect against moisture). The reaction mixture was allowed to stand for 2 hours at room temperature. It was cooled in an ice bath and 100 ml of cold water were added. The solid which formed was filtered and recrystallized from 70 per cent ethanol, giving 4.3 gm of a feathery crystalline solid melting sharply at 155–156°. This represents a 45 per cent yield calculated on allothreonine ester.

$C_7H_{13}NO_2$ (117.14)	Calculated	C 71.93, H 5.55, N 3.36
	Found	" 71.94, " 5.75, " 3.51

An oxalate of the base was obtained by adding a solution of 1.75 gm of oxalic acid in 25 ml of absolute ethanol to a solution of 4 gm of crude reduction product in 100 ml of absolute ethanol. The precipitate was suspended in a liter of ethanol and water was added to the boiling suspension until the solid dissolved. The oxalate separated from the solution on cooling in a nicely crystalline form (m p 199–201°). The yield was 3.7 gm (65 per cent calculated on allothreonine ester).

$C_{10}H_{21}N_2O_8$ (300.31)	Calculated	C 39.96, H 8.00, N 9.33
	Found	" 40.15, " 8.14, " 9.10

*Methyl Ester of DL-Threonine*—This ester was reduced and the crude product converted to the tribenzoyl derivative, as described in "Methyl ester of DL-allothreonine." The crude derivative was a sticky gum which crystallized from aqueous ethanol. On repeated recrystallization from that solvent and from petroleum ether a product was obtained which softened at 127° and melted at 133–135° (20 per cent yield calculated on threonine ester).

$C_{25}H_{43}NO_5$ (417.44)	Calculated	C 71.93, H 5.55, N 3.36
	Found	" 71.85, " 5.67, " 3.39

Mixtures of this material with the 155–156° compound melted in the range 137–148°

*Methyl  $\alpha$ -Aminostearate*—The ester (13 gm) was dissolved in 100 ml of anhydrous methanol. 40 gm of Raney's nickel were added and the mixture was hydrogenated in the usual manner. The reaction was complete when the temperature of the bomb reached 110–115°. The catalyst was filtered and the solvent was removed under reduced pressure. The white solid residue was recrystallized from aqueous methanol and from petroleum ether, giving 7.5 gm (67 per cent yield) of 1-hydroxy-2-amino-octadecane melting at 81–81.5°.

$C_{17}H_{33}NO$ (285.50)	Calculated	C 75.72, H 13.77, N 4.91
	Found	" 75.67, " 13.61, " 4.92

The sulfate of the base was prepared and recrystallized from ethanol (m.p. 205–206° with decomposition). The diacetyl derivative was obtained by warming 1 gm of the base with a mixture of 4 ml of pyridine and 4 ml of acetic anhydride on the steam cone for 20 minutes. The reaction mixture solidified on cooling but the acetyl derivative did not filter readily. Therefore alcohol was added to decompose excess acetic anhydride. The mixture was poured onto iced hydrochloric acid and the derivative was extracted with ether. The ether was removed and the crude product was crystallized from aqueous ethanol, giving 1.0 gm of 1-acetoxy-2-acetamidooctadecane, melting at 98.5–99°.

$C_{22}H_{41}NO_3$ (369.57)	Calculated	C 71.49, H 11.73, N 3.79
	Found	" 71.32, " 11.68, " 3.70

The diacetyl derivative crystallized from several solvents but the crystals in every case were difficult to free of solvent.

*Ethyl Oximinoacetoacetate*—This ester was reduced as described in "Methyl ester of DL-allothreonine". The reduction product gave the tribenzoyl derivative melting at 155–156° (35 to 40 per cent yield).

#### SUMMARY

The methyl esters of DL-allothreonine and DL-threonine are reduced smoothly with Raney's nickel catalyst to 1,3-dihydroxy-2-aminobutane. Allothreonine yields a single isomer, while threonine gives a mixture of the two possible racemic forms. Methyl  $\alpha$ -amino stearate is reduced to 1-hydroxy-2-amino-octadecane in an excellent yield. The application of this reaction to the synthesis of dihydrosphingosine and its isomers is being investigated.

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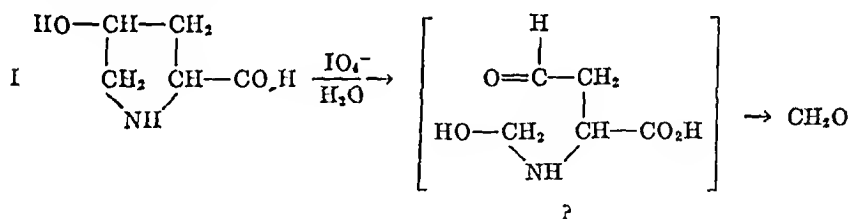
# OXIDATION OF HYDROXYPROLINE BY PERIODATE\*

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(Received for publication, May 12, 1947)

During the course of studies on the periodate oxidation of amino alcohols, it seemed of interest to investigate the behavior of hydroxyproline. This compound contains a 1,2-amino alcohol group but in contrast to other compounds which have been tested the N atom is part of a ring structure, which might possibly interfere with the periodate reaction. Several workers have reported that hydroxyproline yields no acetaldehyde (1, 2) or volatile base (1, 3, 4) under the conditions of the analytical procedures for threonine and total hydroxyamino acids, respectively. However, as far as we can determine from a search of the literature, no study has been made of the reaction of hydroxyproline with periodate and no mention has been made, even in the recent paper of Rees (5), of the possibility that hydroxyproline might interfere with the determination<sup>1</sup> of serine by contributing extra formaldehyde in a normal cleavage of its amino alcohol group.



An investigation of the reaction of hydroxyproline with periodate disclosed that a slow but extensive oxidation occurred, resulting in the consumption of about 4 moles of periodate over a 48 hour period. An extensive series of quantitative experiments established the following facts concerning the products of the reaction.

**Formaldehyde**—As determined by the isolation of the dimedon derivative,

\* The authors wish to express their appreciation to the Abbott Laboratories, Eli Lilly and Company, Parke, Davis and Company, and The Upjohn Company for a grant in support of this work.

† Special research assistant in Chemistry.

<sup>1</sup> In this connection it is interesting to note that serine values for collagen and gelatin by the periodate procedure are erroneously high (6).

0.6 mole of formaldehyde is produced in 1 hour and 0.84 mole in 24 hours, under the conditions of the serine determination (7)

*Acetaldehyde*—Under the conditions of the threonine determination (2), no volatile aldehyde is formed after either 1 or 24 hours

*Ammonia*—Under the conditions of determination of the total hydroxy-amino acids (3), no ammonia is obtained after a 1 hour oxidation period. However, if the oxidizing solution is allowed to stand 24 hours before aeration, a variable but significant amount of ammonia is obtained (0.2 to 0.3 mole)

The exact mechanism of the oxidation of hydroxyproline by periodate has not been worked out. The 1st mole of periodate probably ruptures the pyrrolidine ring, as shown in Equation I. The nature of the subsequent

TABLE I  
*Oxidation of Hydroxyproline with Sodium Metaperiodate*

Time	Periodate consumed per mole hydroxyproline
hrs	moles
0.0	
0.5	0.49
1	0.78
2	1.32
4	2.06
6	2.34
8	2.65
24	3.48
48	3.93

reactions remains to be determined. However, the data now available indicate that hydroxyproline does not interfere with the determinations of threonine or of total hydroxyamino acids. Under the conditions for the periodate determination of serine, hydroxyproline contributes approximately 0.6 mole of formaldehyde, and hence this factor must be considered in evaluating the results obtained for serine with mixtures containing hydroxyproline.

#### EXPERIMENTAL

*Oxidation of Hydroxyproline by Sodium Metaperiodate (8)*—Hydroxyproline (25.4 mg) was placed in a 100 ml volumetric flask, 50 ml of 0.02 M sodium metaperiodate were added, and the solution was diluted to volume. At intervals, 10 ml aliquots of this solution were titrated with 0.01 M sodium arsenite. A blank containing only 0.01 M sodium metaperiodate was titrated at the same time. The course of the reaction is shown in Table I.

*Oxidation of Hydroxyproline according to Procedure for Determination of Threonine and Serine (7)*—A sample of hydroxyproline (11.5 mg) was dissolved in 5 ml of water in a 20 × 2.5 cm Pyrex tube, 5.0 ml of 1 M sodium bicarbonate, 10 ml of 0.1 N sodium arsenite, and 2 ml of 0.5 M paraperiodic acid were added, and the mixture was aerated with carbon dioxide into two receiving tubes containing dilute sodium bisulfite. After 1 hour of aeration, the contents of the receiving tubes were combined and titrated with 0.1 N iodine solution to remove the free bisulfite. Sodium bicarbonate was then added to liberate bound bisulfite. No bound bisulfite was observed.

The oxidation mixture was transferred to a 125 ml flask, and made acid to methyl red with acetic acid. An excess of a 0.4 per cent solution of dimedon was added and the mixture was allowed to stand for 48 hours. The precipitate was collected and dried, giving 9.3 mg (60 per cent yield) of the dimedon derivative melting at 185–186°. In a duplicate experiment, 10.1 mg of hydroxyproline gave 7.8 mg (64 per cent yield) of the dimedon derivative and no volatile aldehyde was produced.

A sample of hydroxyproline (14.0 mg) was dissolved in 5 ml of water, and 5 ml of 1 M sodium bicarbonate, 10 ml of 0.1 N sodium arsenite, and 2 ml of 0.5 M paraperiodic acid were added. The flask was stoppered and allowed to stand for 24 hours when the mixture was transferred to a Pyrex tube and aerated with carbon dioxide into sodium bisulfite for an hour, as in the standard threonine procedure. Again no acetaldehyde (volatile aldehyde) was detected.

The reaction mixture was transferred to a 125 ml flask, acidified, and an excess of 0.4 per cent dimedon solution was added. At the end of 48 hours, 25.6 mg (83.4 per cent yield) of dimedon derivative, melting at 188–189°, were obtained.

*Oxidation of Hydroxyproline according to Procedure for Total Hydroxy-amino Acids (8)*—Two 12.0 mg samples of hydroxyproline were dissolved in 5 ml of water and 15 ml of a saturated solution of potassium carbonate and 2 ml of 0.5 M paraperiodic acid solution were added. These solutions were then aerated into 25 ml of 0.01 N hydrochloric acid for 1 hour. A blank containing only the reagents was treated similarly. At the end of 1 hour, a negligible amount of ammonia was obtained.

Two other samples of 11.0 mg each were dissolved in 5 ml of water and 15 ml of saturated potassium carbonate and 2 ml of 0.5 M paraperiodic acid were added. They were connected to tubes containing 25 ml of 0.01 N hydrochloric acid, and were allowed to stand 24 hours, protected from the atmosphere. The samples, and a blank run concurrently, were then aerated for an hour and 0.3 mole of ammonia was liberated. An additional 0.2 mole of ammonia was obtained on prolonged aeration (5 hours).

Hydroxyproline (20.8 mg) was dissolved in 5 ml of water and a solution of 120 mg of sodium metaperiodate was added. The tube was connected to a tube containing 25 ml of 0.01 N hydrochloric acid and was allowed to stand 24 hours protected from the atmosphere. A blank was run under similar conditions. After 24 hours, 15 ml of saturated potassium carbonate were added, and the mixtures were aerated for 7 hours, yielding 0.55 mole of ammonia per mole of hydroxyproline.

#### SUMMARY

Hydroxyproline undergoes a slow but extensive oxidation by periodate, consuming about 4 moles in 48 hours. Under the conditions of the periodate determination of serine, hydroxyproline yields about 0.6 mole of formaldehyde.

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# BURETTE FOR MICROTITRATION, VERNIER APPLICABLE TO THE CAPILLARY MICRO BURETTE

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(Received for publication, May 12, 1947)

In seeking a suitable micro burette which might be used for analysis of less than 0.02 ml. of whole blood of infants for Ca, in accordance with the method of Sobel *et al.* (1-4), it was apparent that the usual Rehburg type could not be used to measure volumes of acid small enough. A search of the literature revealed that there were four main types of capillary micro burette in use.

The most common type is the Rehburg (5-13) in which the liquid is driven forward before a mercury plunger. The size of the capillary is limited by the fact that in fine capillaries (diameters less than 0.03 cm.) mercury has a tendency to stick. On returning with the plunger, droplets of mercury stick to the capillary wall. The liquid occasionally slides around the mercury and gets behind it.

A second type which was examined is one in which flow is started when the capillary is dipped into the liquid to be titrated and stopped on removal of the capillary into the air or into a pool of mercury. This type has been described by Schwarz, Abel, Heatley, Benedetti-Pichler, and others (14-19). This style is difficult to control at the end-point and the end-point is easily overrun.

In a third type the liquid used is weighed by difference. This method is tedious and not applicable for routine work (20-22).

Recently Scholander has revived interest in the micrometer type in which the plunger is calibrated and the movement of the plunger forward is measured rather than the volume of liquid used (23-28). If a leak develops during a series of routine determinations, large errors may be introduced without the knowledge of the operator, for the actual volume of the liquid is not being measured. It is therefore necessary to calibrate the instrument at regular intervals if it is to be used for routine work.

Up to the present writing these micro burettes have been developed for volumes of from 10 to 50 c. mm.

The authors decided to revert to the Rehburg type and apply the simple principle that the volume of two cylinders is in proportion to the squares of their diameters. A burette was developed in which the mercury would travel in a large capillary and the volume would be measured in a smaller

capillary The general principle of this new type of burette is to carry out the titration as is normally done in the Rehburg burette At the end of the titration, communication is made with a capillary side tube containing the titrating liquid The liquid in the wider capillary is drawn back to the starting mark This liquid comes from the side tube and is measured there This may be observed from Fig 1

### *Apparatus<sup>1</sup>*

*A* is a ground joint which is attached to the mercury screw by means of de Khotinsky cement The mercury is driven no higher than the 0.040

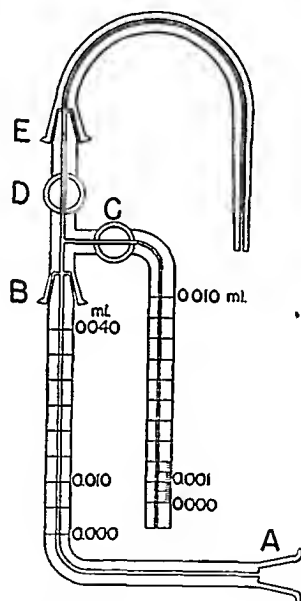


FIG 1 Cross-sectional diagram of micro burette

ml mark shown in Fig 1 *B* is a ground glass joint, which is optional but which makes cleaning easier and allows the removal of the vernier so that the instrument may be used as an ordinary Rehburg burette when so desired This joint may be held together by de Khotinsky cement or by spring clips *E* is a ground glass joint held together by spring clips (not shown) for flexibility of the titrating tip which is easily removed for cleaning

The larger capillary has an internal diameter of approximately 0.05 cm This gives a length of approximately 5 cm for 10 c mm The narrower capillary has an internal diameter of 0.026 cm (approximately) and gives

<sup>1</sup> This instrument may be purchased from the Emil Greiner Company, 161 Sixth Avenue, New York.

a length close to 20 cm. for 10 c. mm. This fine capillary was chosen because it was available in the thermometer tubing stock. Finer tubing could be selected if desired.

In actual practice both capillaries were calibrated by weighing a measured column of mercury in the capillary. The column is moved along to get the calibrations at suitable points and the capillary is then engine-divided. The marks on the wide capillary were complete circles at intervals of 0.005 ml. The narrow capillary was calibrated as shown in Fig. 1. In the fine capillary 0.1 c. mm. divisions were approximately 2 mm. apart.

*C* and *D* are stop-cocks, these must be carefully ground to withstand reduced pressure. The ordinary stop-cocks leak when under a slight vacuum or pressure. A minimum amount of oil-soluble grease should be used. Silicone grease should be avoided because of the difficulty of cleaning the instrument if it enters the capillary tubes.

The stirring is accomplished by a stream of air filtered through ascarite, as shown in Fig. 2. In the photograph the titrating tube and the end-point standard have been pushed aside to show that the capillary delivering the air is below the titrating tip. The air bubbles must hit the titrating tip or serious errors may be introduced, owing to lack of suitable mixing of the fluid delivered. The capillary delivering the air is thrust to the bottom of the tube containing the unknown sample.

### Procedure

*Cleaning and filling*.—The assembly is cleaned by running sulfuric acid-chromic cleaning solution through the instrument, allowing the instrument to stand for 1 hour full of cleaning solution, and rinsing with distilled water with the aid of suction. The apparatus is dried in an oven at 100°. Filtered air is aspirated from time to time through the apparatus until it is dry. The apparatus is assembled and attached to the mercury screw full of mercury in the usual manner. The stop-cocks are lightly greased with petroleum jelly. Stop-cock *C* is closed and the mercury is driven forward until it reaches the tip. The tip is dipped into the titrating solution and the mercury is drawn back until it reaches the zero mark in the wide capillary. From this point on, the mercury is never driven above the 0.040 mark. Stop-cock *D* is closed, *C* is opened, and the solution is driven to the zero mark in the fine capillary. Stop-cock *C* is then closed, *D* is opened, and more solution is drawn in until the mercury is at the zero mark in the wide capillary.

*Titration*.—The stirring is started, stop-cock *C* is closed, and a reading is taken in the narrow capillary. Stop-cock *D* is now opened and the solution is driven forward until the indicator color matches that in the standard tube adjusted to the proper end-point pH. Stop-cock *D* is then closed and

stop-cock *C* is opened. The mercury is drawn back until the level in the wide capillary has returned to the original mark. This fluid comes from the narrow capillary. Stop-cock *C* is now closed and a reading is taken in the narrow capillary. The second titration with a new sample may be begun by opening stop-cock *D* and repeating the procedure above. The first reading in the narrow capillary was taken at the end of the first titration.

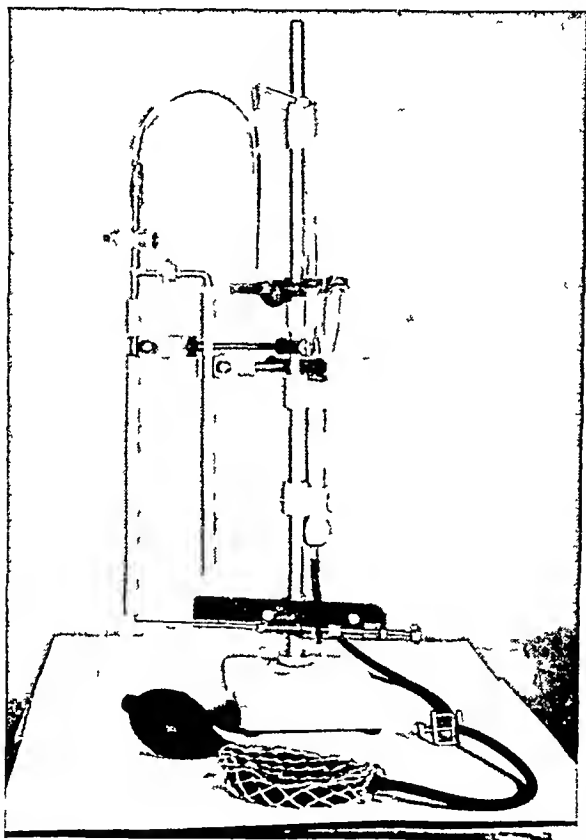


FIG. 2 Photograph of titration assembly

and only one reading need be made at the end of the titration. When more than 0.005 ml. has been used, stop-cock *D* is closed, stop-cock *C* is opened, and the liquid is driven to the next line in the large capillary. Stop-cock *C* is then closed and a reading is taken in the small capillary as the first point in the next titration.

In this manner with a titration volume of about 3 c. mm. approximately twelve titrations may be performed with one filling. When the solution

has approached the 0.010 mark in the large capillary, the tube may be re-filled as before.

If in filling the tube a small bubble of air has lodged behind the mercury in the plunger, it will be noticed that after a titration, with stop-cock *D* closed, the liquid in the narrow capillary will move downward for 1 or 2 divisions when *C* is opened. This jump is a measure of the change in size of the bubble when exposed to the water head as compared to the pull of the narrow capillary. This should be ignored, for the initial and final readings should be taken with the air bubble in equilibrium with the liquid in the fine capillary attached to the wider one. Naturally, elimination of all air bubbles is to be preferred.

TABLE I  
*Results of Ten Consecutive Titrations*

Titration	Mean deviation	Deviation from mean of macrotitration*
<i>c mm</i>	<i>c mm</i>	<i>c mm</i>
2.87	+0.032	-0.005
2.81	-0.028	-0.065
2.80	-0.038	-0.075
2.87	+0.032	-0.005
2.84	+0.002	-0.035
2.87	+0.032	-0.005
2.81	-0.028	-0.065
2.80	-0.038	-0.075
2.84	+0.002	-0.035
2.87	+0.032	-0.005
Mean 2.838		-0.037
Average deviation	±0.0264	

\* Mean of macrotitration converted to *c mm* of acid necessary to titrate 1 ml of 0.003 *N*  $\text{Na}_2\text{CO}_3$  = 2.875 *c mm*

### Results

Table I is typical of the results we have obtained on a consecutive run of ten determinations. The burette was filled with an approximately 1 *N* solution of sulfuric acid. This strong acid was chosen so as to eliminate the end-point error that might be obtained with weaker acid.

The solution titrated was 1 ml of a standard 0.003 *N* sodium carbonate solution. The indicator was made by mixing 5 parts of a 0.1 per cent solution of brom-cresol green in 60 per cent alcohol with 1 part of a 0.02 per cent solution of methyl red in 60 per cent alcohol.

The acid was checked against the sodium carbonate in a macro burette

The macro burette indicated that when the acid was diluted 1000 times 2.875 ml (average of ten determinations, mean deviation  $\pm 1.7$  parts per 1000) were equivalent to 1 ml of the sodium carbonate solution

### DISCUSSION

The values obtained indicate that the reproducibility with the instrument is satisfactory within 1.5 per cent of the 3 c mm measured. This is satisfactory for most biological investigations. However, the mean value of the titration was lower than that obtained from the mean of the macrotitration by 1.3 per cent.

Rieman (19) has suggested that an error of this sort is due to the difference in the value obtained when mercury is used for calibration rather than water. In effect he suggests that a significant error is introduced when one reads the top of the meniscus of the concave mercury and the bottom of the meniscus for water. Thus a unit length of mercury would be less in volume than the same length of water in the capillary. One would need less in the capillary for a titration than the marks indicate. That this could not account for the variations observed can easily be calculated. The volume occupied by the water minus that occupied by the mercury can easily be shown to be twice the difference between the volume of a cylinder (radius and height = the radius of the tube) and the volume of a hemisphere (radius = the radius of the tube). Thus the excess volume might be expressed as  $2\pi(r^3 - \frac{2}{3}r^3) = \frac{2}{3}\pi r^3$ . Since the radius of the capillary is 0.013 cm, the excess volume is equal to 0.0046 c mm. This would account for little more than 10 per cent of the deviation from the macrotitration actually observed.

A more important source of deviation is the choice of end-point. In the macrotitration the acid diluted 1000 times (0.001 N) was titrated against 0.003 N sodium carbonate. This was necessary to obtain reasonable titration values. In the microtitration 1 N acid was used. The end-point was therefore much sharper in the microtitration and was noted sooner than in the macrotitration.

The instrument has all the advantages of the Rehburg type with these added advantages: (1) Volumes less than 3 c mm may be easily measured. (2) The height of the instrument is shortened. If the mercury were driven into the narrow capillary, the capillary would have a height of 100 cm for a capacity equivalent to that of the instrument. The capillary height is one-fifth of that in this instrument. (3) Frequent filling is not necessary since the wider capillary acts as a reservoir. (4) The instrument is flexible for a wide range of titration volumes. Titrations of more than 10 c mm may be carried out by use of the vernier. This volume could then be read to the smallest scale division in the vernier, measuring 0.1 c mm and estimating to 0.01 c mm between marks.

## SUMMARY

A micro burette is described for titration with volumes smaller than 3 cmm. The burette is a mercury plunger type in which the mercury travels in the wider capillary but measurement of liquid volume is made in the narrower capillary. In routine titrations reproducibility within 0.5 per cent is readily attained when the titration is with 3 cmm.

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# FUNCTION OF PYRIDOXAL PHOSPHATE RESOLUTION AND PURIFICATION OF THE TRYPTOPHANASE ENZYME OF *ESCHERICHIA COLI*

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(Received for publication, June 2, 1947)

Since Hopkins and Cole (1) demonstrated the formation of indole from tryptophan by *Bacterium coli*, numerous investigators have attempted to find the mechanism of this reaction (2-5). At least three mechanisms have been suggested. Woods (3) postulated an oxidative degradation of tryptophan to yield indole, carbon dioxide, water, and ammonia, 5 atoms of oxygen being used in the process. Baker and Happold (6) suggested a primary fission into indole and alanine, followed by the oxidation of alanine. Krebs *et al.* (7) proposed that the mechanism involved a preliminary oxidation of the indole ring, followed by oxidation of the side chain to yield *o*-aminophenylacetaldehyde, which condensed to indole spontaneously. The data, however, did not substantiate this view and Krebs was led to state that, while *Escherichia coli* would form indole from *o*-amino- $\beta$ -phenylethanol, via the analogous aldehyde, the mechanism of tryptophanase action very probably did not involve this compound as an intermediate.

Woods (3) and Baker and Happold (6) studied a series of possible oxidative intermediates between tryptophan and indole, and concluded that an unaltered alanine side chain was necessary for tryptophanase action. More recently, Dawson (8) found that mepacrine (atabrine) inhibits tryptophanase, and Dawes, Dawson, and Happold (9) have been able to recover alanine concurrently with indole formation in the presence of mepacrine. They have thus strengthened Baker and Happold's postulate of primary fission to indole and alanine.

In the present study, *Escherichia coli* cells with a very active tryptophanase system have been obtained by growing the culture with aeration. The cells have been vacuum- or acetone-dried to yield cell preparations which contain most of the activity present in the living cells. The enzyme is stable in these preparations and may be obtained in a cell-free state by autolysis. The resolution and purification of the enzyme have been accomplished by precipitation of the cell-free extracts with ammonium sulfate and by calcium phosphate adsorption. Pyridoxal phosphate will reactivate the resolved enzyme, thereby adding tryptophanase to the group of vitamin B<sub>6</sub> enzymes.

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The products of the action of these purified preparations, as well as of the dried cell preparations, are indole, pyruvate, and ammonia, in an equimolar ratio

The purified tryptophanase enzyme does not deaminate alanine or serine, thus neither of these is an intermediate in the tryptophanase reaction

### Methods

*Culture*—The Crookes strain of *Escherichia coli* from the departmental collection was used. With this culture a very active tryptophanase enzyme was obtained by growth in a medium composed of 1 per cent tryptone, 1 per cent yeast extract, 0.5 per cent  $K_2HPO_4$ , and 0.1 per cent glucose. The medium was dispensed in 200 ml amounts in 500 ml Erlenmeyer flasks, inoculated, incubated 4 to 6 hours at 30°, then placed in a mechanical shaker (approximately a hundred 3 inch strokes per minute) and incubated for an additional 18 to 20 hours. The cells were harvested with a Sharples centrifuge. The cell paste from 6 liters of medium was washed with 250 ml of water, centrifuged, resuspended in 15 to 20 ml of distilled water, and dried *in vacuo* over drierite to yield about 10 gm of dried cells. Acetone-dried cells were prepared by pipetting the washed cell suspensions into 10 volumes of ice-cold acetone. The cells were collected on a Buchner funnel and washed with ether. Both the vacuum-dried and acetone-dried preparations contained two-thirds or more of the tryptophanase activity present in the living cells, the enzyme being stable in the dried state.

*Tryptophanase Determination*—Tryptophanase activity was determined by measuring indole formation. The usual assay was performed in 2 ml volume containing the following: 0.2 ml of 1 M phosphate buffer, pH 8.3, 20  $\gamma$  of barium pyridoxal phosphate, 50 to 500  $\gamma$  of the cell preparations described above, 2 mg of L-tryptophan. The enzyme, buffer, and co-enzyme were incubated at 37° in a volume of 1.8 ml for 10 minutes. The substrate (0.2 ml) was added, the reaction allowed to proceed 10 minutes, and then stopped with 0.2 ml of 100 per cent trichloroacetic acid. The indole was extracted by shaking with 2 ml of toluene, and a portion of the toluene layer was removed for analysis.

*Analytical Methods*—Indole was determined by Ehrlich's method, modified as follows. From 0.2 to 1 ml of toluene layer, depending on the level of indole expected, was pipetted into a colorimeter tube and 1 ml of 5 per cent *p*-dimethylaminobenzaldehyde in ethyl alcohol was added. The tubes were filled to the 10 ml line with an acid alcohol solution (1 liter of ethyl alcohol plus 80 ml of concentrated sulfuric acid), allowed to stand 10 minutes, and read in an Evelyn colorimeter, with the No. 540

filter. With this method, indole can be determined over a range of 1 to 15  $\gamma$ , the method may be used to 20  $\gamma$  without great deflection from linearity.

*Pyruvate*—For most determinations, the direct method of Friedemann and Haugen (10) with 2,4-dinitrophenylhydrazine was used. The identity of the pyruvate was established by the toluene extraction method, the quantitative data agreeing with the direct method.

*Ammonia*—Ammonia was determined by nesslerization after distillation from a Pregl still (11). The reaction mixture was deproteinized with trichloroacetic acid, neutralized to nearly pH 7, and pipetted into the still. 2 ml of a borate buffer (12) were added and the sample steam-distilled for 5 minutes, about 6 ml of distillate being collected. The distillate was nesslerized by adding 2 ml of Johnson's reagent (13) and 1 ml of 6 N alkali, and then diluted to 10 ml. After 10 minutes the color was read in the Evelyn colorimeter with the No. 490 filter.

### Results

With the dried cell preparations, it was found that the quantity of indole formed was proportional to the cell concentration only at the lower levels (Fig. 1). The lack of proportionality of indole production at the higher cell concentrations was later shown to be due to the inhibitory effect of indole, as previously described by Fildes (2). The enzyme could be assayed reasonably well, however, over a range of cell concentrations from 50 to 300  $\gamma$ , equivalent to 1.5 to 12  $\gamma$  of indole formed in a 10 minute incubation period. As shown in Fig. 2, the rate of indole production by a given cell concentration also decreased with time. Inasmuch as the cell preparations were sufficiently active to give accurate analytical values within a 10 minute period, there was no need for a longer incubation time.

The dried preparations did not show an oxygen uptake with tryptophan, and so no effort was made to run the experiments anaerobically. An attempt to determine the products of tryptophanase action showed that pyruvate was formed in approximately equimolar ratio to the indole formed. The preparations contained serine and alanine deaminases, which also formed pyruvate, thus one of these might be an intermediate in the tryptophanase reaction. The alanine would be considered as a possible intermediate in view of the work of Dawes, Dawson, and Happold (9), and serine might be considered in view of the formation of tryptophan from serine and indole by the *Neurospora* enzyme (14, 15). In order to determine whether either of these was an intermediate in the reaction, conditions were sought in which the tryptophanase was active whereas the deaminases were not.

In view of the function of pyridoxal phosphate as the coenzyme of tryptophan synthesis with the *Neurospora* enzyme (15), the possibility of

its function in the tryptophanase reaction was obvious. Attempts to demonstrate the action of pyridoxal phosphate with the dried preparations resulted in approximately 50 per cent stimulation of the rate of indole formation. Thus, even in the dried preparations, the enzyme system which forms indole from tryptophan was partially resolved and could be activated by pyridoxal phosphate as the coenzyme.

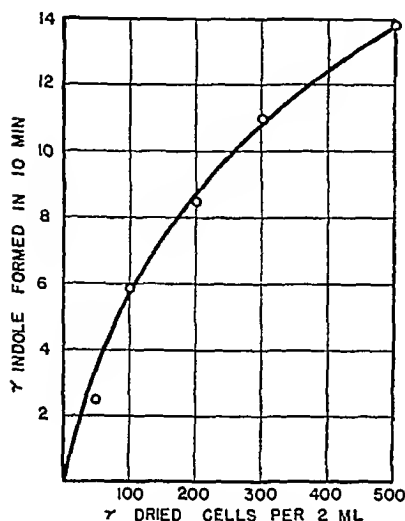


FIG 1

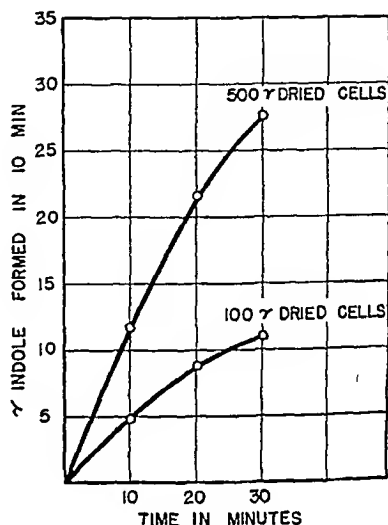


FIG 2

FIG 1 Tryptophanase activity of dried cell preparation. 2 ml reaction volume containing 0.2 ml of 1 M phosphate buffer, pH 8.3, 20 γ of pyridoxal phosphate (barium salt), dried cells as indicated, water to 1.8 ml, let stand 10 minutes at 37°, add 0.2 ml (2 mg) of L-tryptophan, incubate 10 minutes at 37°.

FIG 2 Tryptophanase activity and incubation time. Conditions are the same as in Fig 1.

### *Enzyme Purification and Resolution*

10 gm of an acetone-dried preparation were evenly suspended in 500 ml of water in a Florence flask and placed in a cold room overnight and allowed to freeze. The next morning the flask was removed from the cold room and placed in a 37° water bath, where thawing and autolysis were allowed to proceed for 2 hours. The cell debris was removed by centrifugation and the supernatant treated at room temperature, with an equal quantity of saturated ammonium sulfate neutralized to a pH of about 8.5. Upon standing in the refrigerator for a short time, the precipitate flocculated and was removed by centrifugation. The supernatant was saturated with solid ammonium sulfate and 7 ml of 0.01 M sodium cyanide

added (final concentration 0.0001 M). The solution was allowed to stand in the refrigerator until the precipitate flocculated, and was then centrifuged. The precipitate was suspended in 270 ml of water and the insoluble matter removed by centrifugation. The supernatant solution was again treated with ammonium sulfate to 55 per cent saturation and the small quantity of precipitate which formed was discarded. The enzyme was then precipitated by adding ammonium sulfate to 68 per cent saturation and the precipitate removed by centrifugation. This precipitate, which contained about 10 per cent of the enzyme originally present in the cells, was suspended in 50 ml of water. Pilot experiments were performed to determine the concentration of calcium phosphate gel necessary just to adsorb the enzyme, and the indicated amount was added with mixing and allowed to stand 10 minutes. The phosphate gel was

TABLE I

*Pyruvate formation by Cell Preparation and by Purified Enzyme*

The conditions are the same as in Fig. 1

Substrate	Pyruvate formed	
	0.5 mg cell preparation	0.05 ml cell free enzyme
	$\gamma$	$\gamma$
L-Tryptophan	18.3	42.5†
“ without coenzyme	12	0
DL Alanine	1.0	0.3
DL Serine	8.1	0
L Serine*	5.0	0.1

\* We wish to thank Dr. J. S. Fruton for a supply of L serine.

† 50  $\gamma$  of indole formed, without coenzyme no indole formed.

collected by centrifugation and washed with five 250 ml portions of distilled water, after which the enzyme was eluted from the gel with 50 ml of 1 M phosphate buffer, pH 6.0. The enzyme at this stage of purification was completely resolved and free from serine and alanine deaminases (see Table I).

#### *Characteristics of Tryptophanase*

The influence of tryptophan concentration on the reaction rate is shown in Fig. 3. With the cell-free enzyme, the half maximum rate is obtained with 35  $\gamma$  of tryptophan per ml (Michaelis constant (16),  $K = 2.5 \times 10^{-5}$  mole per liter). With the dried cells, a somewhat higher substrate concentration is required, the rate of indole formation dropping sharply below 200  $\gamma$  of tryptophan per ml.

The coenzyme saturation curve for tryptophanase is shown in Fig 4, the half saturation concentration of barium pyridoxal phosphate being  $0.9 \gamma$  per ml (Michaelis constant,  $K = 2.1 \times 10^{-6}$  mole per liter). Thus the dissociation constant of the pyridoxal phosphate-tryptophanase complex approximates that of the glutamic-aspartic transaminase,<sup>1</sup>  $K = 1.5 \times$

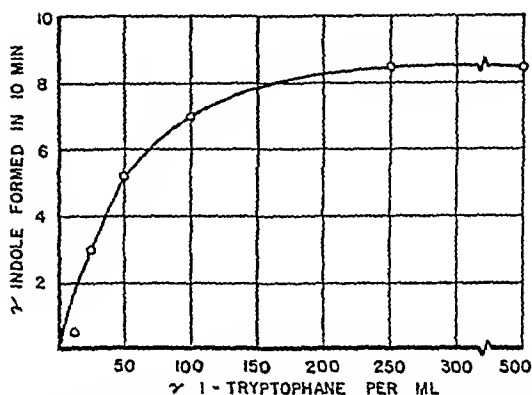


FIG 3 Substrate saturation curve for tryptophanase Conditions are the same as in Fig 1, 0.03 ml of purified enzyme

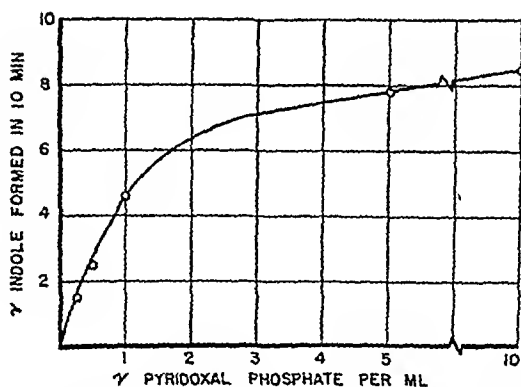


FIG 4 Coenzyme saturation curve for tryptophanase Conditions are the same as in Fig 1, 0.03 ml of purified enzyme

$10^{-6}$ , but higher than that of the tyrosine decarboxylase,<sup>2</sup>  $K = 1.5 \times 10^{-8}$  mole per liter. Both the tryptophanase and the transaminase reactions are run at neutral reaction, but the tyrosine decarboxylase is run at an acid pH.

<sup>1</sup> O'Kane, D. E., and Gunsalus, I. C., *J. Biol. Chem.*, in press

<sup>2</sup> Gunsalus, I. C., and Umbreit, W. W., unpublished data

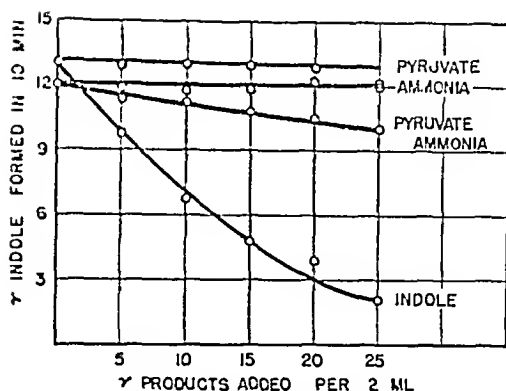


FIG 5 Influence of products upon tryptophanase activity Conditions are the same as in Fig 1 0.03 ml of purified enzyme, indole pyruvate and ammonia, added in the quantities indicated, before the enzyme is added

TABLE II

*Inhibition of Tryptophanase by NaCN*

Conditions are the same as in Fig 1, 0.03 ml of purified enzyme, cyanide added after enzyme and coenzyme

Concentration of cyanide	Indole formed	Per cent inhibition
$\mu$	$\gamma$	
$10^{-1}$	0.0	100
$10^{-2}$	1.0	95
$10^{-4}$	5.2	70
$10^{-5}$	11.5	35
$10^{-6}$	17.5	0
0	17.5	

TABLE III

*Products of Tryptophanase Reaction*

Conditions 7 ml, reaction volume, 0.7 ml of 1 M phosphate buffer, pH 8.3, 70  $\gamma$  of pyridoxal phosphate (barium salt), 0.3 ml of purified enzyme Water to 6.3 ml, let stand 10 minutes, 37°, 0.7 ml (7 mg) of L tryptophan added and incubated 10 minutes

Experiment No	Micromoles of products formed		
	Indole	Pyruvate	Ammonia
2057	0.92	0.99	0.13
2101	1.37	1.09	1.12
2102	1.44	1.33	1.34
2103	1.02	1.07	1.03
2103a	0.82	1.25	1.28
Molar ratio (average)	1.00	1.05	1.05

Tryptophanase is sensitive to indole accumulation, as indicated by the cell preparations, Figs 1 and 2, and as shown with the cell-free enzyme, Fig 5. The presence of pyruvate plus ammonia has a slight influence.

The enzyme is sensitive to cyanide (Table II). This sensitivity, however, may not indicate an iron catalyst but a reaction between cyanide and the free carbonyl group of pyridoxal phosphate. A similar sensitivity of pyridoxal phosphate-containing enzymes to cyanide is mentioned by Blaschko (17) and by Gale (18) for the animal and bacterial decarboxylases respectively.

### *Products of Tryptophanase Reaction*

The tryptophanase reaction, as catalyzed by the dried cell preparations and by the partially purified enzyme, can be expressed by the following equation: tryptophan  $\rightarrow$  indole + pyruvic acid + ammonia. Analyses of the products formed in several experiments with the purified enzyme are shown in Table III.

### DISCUSSION

From the data presented, it is apparent that the tryptophanase system of *Escherichia coli* is not the reversal of the indole and serine condensation which leads to tryptophan with the *Neurospora* enzyme. With purified tryptophanase the products are indole, pyruvate, and ammonia. Serine and alanine do not yield pyruvate, and are not, as such, intermediates in the reaction. The intermediates postulated by various investigators (3, 5, 7), with the possible exception of amino acrylic acid (12), do not appear to be involved.

### SUMMARY

Tryptophanase has been obtained in a cell-free state from *Escherichia coli* and has been partially purified.

The enzyme has been resolved and shown to require pyridoxal phosphate as the coenzyme.

The enzyme preparation catalyzes the breakdown of tryptophan according to the following reaction: tryptophan  $\rightarrow$  indole + pyruvic acid + ammonia. No oxidation occurs in the process, nor does alanine or serine occur as an intermediate.

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# PTEROYLASPARTIC ACID, AN ANTAGONIST FOR PTEROYLGLUTAMIC ACID

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(Received for publication, June 7, 1947)

Pteroylaspartic acid, N-[4-[(2-amino-4-hydroxy-6-pteridyl)methyl]-amino]benzoyl]aspartic acid, has been found to be an antagonist of pteroylglutamic acid and certain of its derivatives for several species. The present communication reports the preparation of the compound and summarizes the experiments demonstrating its antipteroylglutamic acid activity.

## EXPERIMENTAL

*Preparation of Pteroylaspartic Acid Aspartic Acid*—A mixture of Bacto-asparagine (250 gm), water (740 ml), and concentrated hydrochloric acid (270 ml) was refluxed for 3 hours, and then cooled to 30°. To this solution were added 112 ml of 28 per cent ammonium hydroxide solution with good stirring. The precipitate of aspartic acid was collected and recrystallized from 3800 ml of water. Yield, 169 gm.

*p-Nitrobenzoylaspartic Acid*—To a well stirred solution of aspartic acid (80 gm) and sodium hydroxide (60 gm) in 600 ml of water were added 140 gm of *p*-nitrobenzoyl chloride and 600 ml of 2 N sodium hydroxide solution during about 45 minutes. The temperature of the reaction mixture was not allowed to rise above 35°. After being stirred for 1 hour the solution was acidified with 164 ml of concentrated hydrochloric acid. The precipitate of *p*-nitrobenzoic acid (48 gm) was separated by filtration, and the filtrate (1700 ml) was adjusted to pH 1.5, seeded, and chilled for several hours. The crystalline product was collected, washed with water, and dried. Weight 110 gm, m.p. 145–147°.

$[\alpha]_D^{20} = +26.3^\circ$  (2% solution in 1 N sodium hydroxide)

*p-Aminobenzoylaspartic Acid*—A mixture of *p*-nitrobenzoylaspartic acid (100 gm), glacial acetic acid (700 ml), water (300 ml), and platinum oxide catalyst (1.5 gm) was heated to 45° and shaken with hydrogen at atmospheric pressure until the theoretical amount of hydrogen had been absorbed. The catalyst was removed by filtration and the filtrate evaporated nearly to dryness *in vacuo*. The residue was then suspended in glacial acetic acid,

collected, and washed with acetic acid, acetone, and ether. The anhydrous product weighed 71 gm. This crude product was extracted with 500 ml of boiling acetone and then collected on the filter and washed with acetone, ether, and petroleum ether. Yield, 45 gm, m p 171-173°. The acetone extraction resulted in considerable loss and is probably unnecessary.

$$[\alpha]_D^{25} = -10.7^\circ \text{ (2\% solution in 1 N hydrochloric acid)}$$

A sample was recrystallized from glacial acetic acid for analysis.

$C_{11}H_{12}O_5N_2$	Calculated	C 52.35, H 4.76, N 11.11
	Found	" 52.11, " 5.29, " 11.04

*Pteroylaspartic Acid*—A solution of 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride<sup>1</sup> (68.5 gm) and *p*-aminobenzoylaspartic acid (40 gm) in 4 liters of water was adjusted to pH 4.0 by the addition of sodium hydroxide solution. Then with good stirring, a solution of 2,3-dibromopropionaldehyde (69 gm) in 1500 ml of ethanol was added during about 2 hours. The pH was maintained at 4.0 by the addition of sodium hydroxide solution. After the addition of the dibromopropionaldehyde, the solution was stirred for an additional 45 minutes and the precipitate was then collected on the filter, washed with water, alcohol, acetone, and ether, and dried. Weight, 71 gm.

Chemical assay (2) of the product was 19.2 per cent. Yield, 13.65 gm of pteroylaspartic acid.

*Purification of Pteroylaspartic Acid. Precipitation of Impurities with Barium-Ethanol*—71 gm of the crude pteroylaspartic acid were dissolved in 50 liters of 0.2 N sodium hydroxide solution. Barium chloride was added to 0.2 N and ethanol to a concentration of 2 per cent. Filter-Cel was added and the solution clarified. The excess barium was removed from the filtrate by the addition of sulfuric acid and the precipitated barium sulfate removed by filtration. There were 9.068 gm of pteroylaspartic acid by chemical assay in the filtrate.

*Precipitation at pH 3.0*—The solution was neutralized to pH 7.0 and concentrated under nitrogen to 20 liters. The precipitate that formed was removed by filtration and discarded. The solution was adjusted to pH 3.0, thoroughly chilled, and collected with the aid of Filter-Cel. The precipitate was extracted with 12 liters of 0.2 N sodium hydroxide solution. Yield, 5.92 gm by chemical assay.

*Treatment with Charcoal*—The alkaline extract was stirred with 7 gm of norit A<sup>2</sup> for 15 minutes and the norit A removed by filtration. The

<sup>1</sup> 2,4,5-Triamino-6-hydroxypyrimidine (1) was dissolved in dilute hydrochloric acid and crystallized by the addition of concentrated hydrochloric acid to 6 N.

<sup>2</sup> Activated charcoal, Pfanstiehl Chemical Company, Waukegan, Illinois.

filtrate was adjusted to pH 3.0, chilled thoroughly, and the precipitate collected on a Sharples centrifuge.

**Formation of Magnesium Salt**—The precipitate was suspended in 1 liter of water and excess magnesium oxide added. The solution was heated to 65° for 15 minutes and filtered. The hot solution was treated with norit A until the brownish pigments were removed. The light yellow solution was chilled and the needles of magnesium pteroylaspartate were collected and dried. Yield, 1.133 gm.

$C_{14}H_{14}N_2O_6Mg \cdot \frac{1}{2}H_2O$	Calculated	C 45.14, H 3.34, N 20.48, Mg 7.62
	Found	" 44.93, " 3.89, " 20.30, " 7.66

The compound is quite stable as the magnesium salt. Attempts to crystallize the free acid out of hot water previously adjusted to pH 3.0 caused the compound to break down partially into pteric acid and aspartic acid.

**Bacterial Inhibition**—The antipteroylglutamic acid activity of pteroylaspartic acid was determined with *Lactobacillus casei* and *Streptococcus faecalis* R. *S. faecalis* R was grown on the medium of Tepley and Elvehjem (3). For the experiments with *L. casei*, alanine and asparagine were omitted from the medium. The incubation times for *L. casei* and *S. faecalis* R were 16 and 72 hours, respectively, at 32–33°.

The inhibition index is defined as the ratio of the amount of inhibitor to metabolite that will produce half maximum inhibition. The general procedure is to add a given amount of the metabolite to a set of tubes and to add varying amounts of the inhibitor. An inhibition curve is plotted and from this the point of half maximum inhibition is obtained.

As an example of an organism that does not require preformed pteroylglutamic acid, *Escherichia coli* was used. The bacterium was grown on a medium containing 0.5 per cent peptone, 0.3 per cent beef extract, and 0.1 per cent dextrose.

**Chick and Rat Experiments**—For the inhibition studies with the chick, day-old New Hampshire red chicks were placed on the basal diet of Hutchings *et al.* (4). All preparations of the metabolite and inhibitor were injected intramuscularly daily. For maximum stability of the inhibitor, the magnesium salt was dissolved by gentle heating in water containing a small amount of sodium bicarbonate. After cooling to room temperature, the compound was injected immediately.

In the rat experiments the basal diet of Spicer *et al.* (5) was used. The effect of the inhibitor was also studied on the above diet supplemented with 0.5 per cent carboxysulfathiazole. The general procedure was to place 21 day-old rats on the diets and inject intramuscularly varying amounts of the inhibitor daily. Growth and complete blood counts were followed.

the inhibitor caused a marked decrease in the growth rate, and the hemoglobin level was decreased to a value approaching that for the basal diet. It is apparent that the inhibitor antagonizes the growth effects of pteroylglutamic acid in a ratio of 500:1 by weight.

*Rat*—The addition of the inhibitor in amounts up to 1.5 mg. per day per rat caused no significant decrease in the growth rate or the appearance of any symptoms characteristic of a pteroylglutamic acid deficiency on either of the diets used.

#### DISCUSSION

From the foregoing experiments it is apparent that pteroylaspartic acid is an effective inhibitor in the utilization of pteroylglutamic acid for growth by *Lactobacillus casei*, *Streptococcus faecalis* R, and the chick. With *Streptococcus faecalis* R the compound was more effective as an inhibitor of the utilization of pteric acid and the pteroyldiglutamic and pteroyltriglutamic acids. These latter compounds were not tested with *Lactobacillus casei* or the chick.

The inhibitor was not effective in interfering with the metabolism involving pteroylglutamic acid or derivatives in *Escherichia coli*. In the rat the inhibitor was not effective in interfering with the utilization of pteroylglutamic acid.

#### SUMMARY

Pteroylaspartic acid was synthesized from *p*-aminobenzoylaspartic acid, 2,3-dibromopropionaldehyde, and 2,4,5-triamino-6-hydroxypyrimidine. The compound is an antagonist for pteroylglutamic acid with *Lactobacillus casei* and the chick. With *Streptococcus faecalis* R the inhibitor effectively prevents the utilization of pteric acid, pteroylglutamic acid, pteroyl- $\gamma$ -glutamylglutamic acid, and pteroyl- $\gamma$ -glutamyl- $\gamma$ -glutamylglutamic acid. In all instances the inhibition is of a competitive nature.

We are especially indebted to Miss E. Boggiano for the many microbiological assays, to Miss H. Ritter for the hemoglobin determinations, and to Mr. L. Brancone and coworkers for the microanalyses.

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## A FUNCTION FOR BIOTIN

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(Received for publication, June 9, 1947)

During studies on the metabolism of aspartic acid in *Escherichia coli* it became evident that cells harvested from a complex medium rapidly lost activity and that the lost activity could be restored by the addition of a mixture of the known members of the vitamin B complex. By restricting the conditions of measurement the active agent among these vitamins proved to be biotin, and by separating the various reactions concerned in the complex the biotin was shown to function in oxalacetate decarboxylation. However, some of the evidence available indicates that the natural substrate for this enzyme is not oxalacetic acid as such but a material resembling it, perhaps a phosphorylated oxalacetate. This study represents an unusual mode of obtaining a vitamin deficiency and reveals an unexpected property of biotin.

That there existed a relationship between biotin and aspartic acid was shown by Koser, Wright, and Dorfman (1) and Stokes, Larsen, and Gunness (2). The latter workers investigated several of the reactions concerned in the metabolism of aspartic acid and found that biotin was not concerned with transamination. A suggestion that biotin was concerned with the formation of oxalacetate was reported, since oxalacetic acid partially replaced the biotin requirement for some organisms in an aspartic-deficient medium. Winzler, Burk, and du Vigneaud (3) found that biotin-deficient yeast cells were markedly stimulated by biotin to take up ammonia.

### Methods

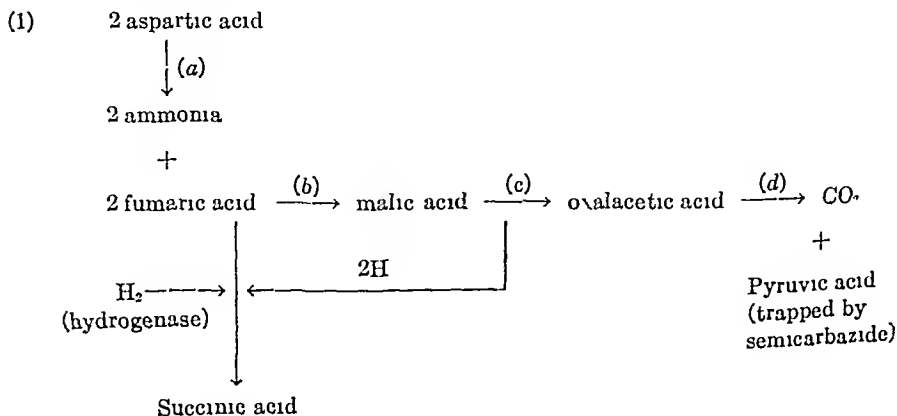
The culture used was a strain of *Escherichia coli* obtained originally from Giatra in Belgium and carried in the laboratories of Professor P. F. Clark, to whom we are indebted for a transplant. It was grown for 16 to 18 hours in 1 per cent pepticase, 1 per cent yeast extract, 0.5 per cent  $K_2HPO_4$ , and 0.3 to 0.6 per cent glucose, at 27°. The cells were harvested by centrifugation, washed once with water or saline, and suspended in M phosphate at pH 4 (0.5 to 1 mg. of N per ml.). Such suspensions will liberate carbon dioxide from added aspartic, fumaric, or malic acid under anaerobic con-

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ditions in the presence of 0.01 to 0.02 M semicarbazide, which prevents the reaction from proceeding beyond the pyruvate stage. Active cell suspensions are maintained for several hours upon refrigeration but, when incubated at 37° for from 15 to 60 minutes (the time required varies somewhat from suspension to suspension), such cells manifest markedly decreased rates of carbon dioxide evolution upon the addition of aspartic, fumaric, or malic acid. Deficient cells are therefore obtained by exposing them at pH 4 in M phosphate to 37° for a relatively short period. Conventional Warburg techniques (4) were used throughout, and all measurements were made at 37°. The biotin used was the free form. Ammonia was measured, as previously described (4), after centrifugation to remove the cells. Succinate was measured with succinoxidase (4). Pyruvate was measured after destroying the semicarbazide by heating with nitrous acid. To the sample (0.1 to 0.5 ml in 1 per cent  $\text{H}_2\text{SO}_4$ ) was added 0.5 ml of 1 per cent  $\text{KNO}_2$ , and the mixture heated in a steam bath for 30 minutes. Then 0.1 ml of 10 per cent urea was added (to destroy any nitrite remaining) and allowed to react for 10 minutes, and 0.1 ml of 100 per cent trichloroacetic acid and 0.5 ml of fresh 2,4-dinitrophenylhydrazine reagent of Friedemann and Haugen (5) were added and the mixture allowed to react for 5 minutes. 3 ml of toluene were then added, dispersed through the mixture by shaking, and the layers allowed to separate. 2 ml of the toluene (upper) layer were removed to a colorimeter tube and 3 ml of 1.5 per cent KOH in 95 per cent alcohol were added, and after 10 minutes the color was read in an Evelyn photoelectric colorimeter with a No. 515 filter. The range of this method is from 0 to 30  $\gamma$  of pyruvate.

### Reaction

The degradation of aspartic acid in this organism may be represented by the accompanying series of reactions





This series of reactions operates at pH 4 and, except when otherwise stated, the studies were made at this pH. This is of some advantage in that the low pH slows down the further breakdown of pyruvate, thus minimizing certain complications of measurement. In order to demonstrate certain steps in the reaction chain, it was sometimes convenient to work at a higher pH.

The over-all reaction, which may not always run to completion, produces 2 ammonia, 1 succinate, and 1 pyruvate per carbon dioxide produced from aspartic acid and a similar balance of products, with the exception of ammonia from malate. These results are incorporated in equation (1) and evidence for the intermediate steps are given below.

The aspartic deaminases (b) (reaction (a)) are normally operative at a slightly alkaline pH, with this organism deamination was almost as rapid at pH 4 as at pH 6 or 8. At pH 4 under anaerobic conditions the production of ammonia was 2 moles per mole of carbon dioxide, as required by equation (1). Evidence that the product of deamination was fumaric acid was obtained by the use of a vacuum-dried preparation in which reaction (c) was eliminated and reaction (b) (fumarase) was weak. These preparations contained an active hydrogenase which would reduce fumarate with molecular hydrogen. Measurements of hydrogen uptake would thus readily distinguish between malic and fumaric acids. At pH 5, 0.3 M phosphate, 10 mg of preparation, with KOH in the center well, 224 microliters of substrate, and an atmosphere of  $H_2$ , the rate of hydrogen uptake was L-aspartic acid  $Q_{H_2} = 24$ , fumaric acid  $-24$ , L-malic acid  $-6$ . Living cells, in contrast to vacuum-dried preparations, contain an active fumarase (reaction (b)), since malate is as rapidly reduced by molecular hydrogen as is fumarate. At pH 5, 0.3 M phosphate, 4 mg of cell nitrogen, KOH in center well, 224 microliters of substrate in an atmosphere of  $H_2$ , the rate of hydrogen uptake was L-aspartic acid  $Q_{H_2} (N) = 300$ , fumaric acid  $-264$ , L-malic acid  $-246$  (total hydrogen uptake 229.7, 216.1, and 227.6 microliters respectively). Further, the rate of carbon dioxide evolution is the same, whether one adds aspartic or malic acid (the rates with fumarate being somewhat variable because of a permeability effect).

Malic dehydrogenase (reaction (c)) was readily demonstrated at pH 7.3 by the reduction of methylene blue in the presence of cyanide essentially as described by Green and Williamson (7). Other methods of demonstrating this enzyme at pH 4 are apparent in the data to be presented. A heat-labile oxalacetate decarboxylase (reaction (d)) was demonstrated, as will also be evident in subsequent data.

#### EXPERIMENTAL

When cells obtained as previously described were held in M/3 phosphate buffer, pH 4, at 37° for intervals of 45 to 60 minutes, the rate of carbon di-

oxide production from added aspartic acid was markedly decreased. This diminished activity could be restored by the addition of a mixture of vitamins, and the important agent in this mixture, as is seen in Table I, was biotin. Biotin was therefore concerned somewhere in a series of reactions given in equation (1). The loss of activity occurs in the presence or absence of semicarbazide, and at 25–28° at a somewhat slower rate than at 37°. The biotin additions may be made before or after the aging process with the same results.

TABLE I

*Carbon Dioxide Production from Aspartic Acid*

Cells of *Escherichia coli* grown as described in the text, suspended in 1 M phosphate at pH 4 and held at 37° for intervals indicated. Reaction run in 0.3 M phosphate, 0.01 or 0.02 M semicarbazide, pH 4, 37°, about 0.5 mg of cell nitrogen per cup, N, aspartic acid adjusted to pH 4 tipped at 0 time. Reaction volume 3 ml. Exposure to pH 4 in the absence of additions. Levels of biotin indicated are millimicrograms of biotin per 3 ml.

Experiment No	Time of exposure to 37°, pH 4	$Q_{CO_2}$ (A)			
		No additions 0 biotin	Vitamins * 5 m $\gamma$ biotin	Vitamins less biotin,† 0 biotin	Biotin, 500 m $\gamma$
407	60	71	334		
409	45	18	274	25	273
411	45	117	223		230
415	60	8	202		259
421	60	24	177	20	178

\* Vitamins added per cup: riboflavin 1  $\gamma$ , nicotinic acid 2.5  $\gamma$ , pantothenic acid 1  $\gamma$ , pyridoxal 10  $\gamma$ , folic acid 5  $\gamma$ , thiamine 5  $\gamma$ , *p*-aminobenzoate 5  $\gamma$ , biotin 5 m $\gamma$ . We are indebted to Merck and Company, Inc., for some of the vitamins and to the Lederle Laboratories Division, American Cyanamid Company, for the synthetic folic acid.

† Vitamins less biotin at the same levels as above, but with biotin omitted from the mixture.

With malate as the substrate (Table II), the same biotin stimulation of deficient cells was obtained. Occasionally, as in Experiment 427-a, deficiency of factors in addition to biotin is evident inasmuch as the entire vitamin mixture was effective, but biotin itself would not suffice. It may also be noted that the levels of biotin required are relatively low, 5 m $\gamma$  being adequate and lower levels such as 0.5 m $\gamma$  (Experiment 444) sufficing. Inasmuch as, with malate as the substrate, reaction (a) (aspartic decarboxylase) was not operative, biotin was therefore concerned with the reactions of equation (1) other than (a).

Evidence that the biotin effect resides in (d) (oxalacetate decarboxylase)

was obtained in two ways. First, as is indicated in Table III, deficient cells which can be stimulated with biotin show no biotin effect when malate is oxidized in the presence of large quantities of cyanide to bind the oxalacetate. In order to observe the second system, an artificial hydrogen

TABLE II  
*Carbon Dioxide Production from Malic Acid*

Conditions as in Table I, except that malic acid adjusted to pH 4 was used as a substrate rather than aspartic acid. Numbers in parentheses indicate millimicrograms of biotin supplied per 3 ml if this is different from 5 mγ per 3 ml.

Experiment No	Time of exposure to 37°, pH 4	Other treatments	Q <sub>CO<sub>2</sub></sub> (N)			
			No additions, 0 biotin	Vitamins, 5 mγ biotin	Vitamins less biotin	Biotin 5 mγ
411	45	Cells at 0-5°, 4 hrs	109	206		199 (500)
420	60		34	205	15	234 (500)
424	30		0	86	0	79 (50)
427	30		183	264		171
436	10		46	166		149
441	15	Cells at 0-5°, 4 hrs	5	103	10	92
413 a	30	" grown in 0.3% glucose	14	99		103 (0.5)
443 b	30	" " " 0.15% "	6	45		99
						45

TABLE III  
*Decomposition of Malate to Oxalacetate or to Carbon Dioxide*

(a) Malate to CO<sub>2</sub> in N, conditions as in Table II, cells exposed for 30 minutes before use, about 0.8 mg of cell nitrogen per cup. (b) Malate to oxalacetate, value given is O<sub>2</sub> uptake, cells at pH 4 in refrigerator 60 minutes, then at 37° for 30 minutes, reaction run in 0.3 M phosphate, 0.02 M semicarbazide, pH 4, 37°, about 0.8 mg of cell nitrogen per cup, gas phase air, 0.1 M NaCN, methylene blue.

Experiment No	Conditions	Q (N)		
		No additions, 0 biotin	Vitamins, 5 mγ biotin	Biotin, 50 mγ
456	(a) Malate to CO <sub>2</sub>	42	91	89
	(b) " " oxalacetate	146	146	146
461	(a) " " CO <sub>2</sub>	32	56	56
	(b) " " oxalacetate	127	127	

carrier (methylene blue) was employed, since the cyanide present inhibited the normal respiratory mechanisms of this organism. This suggests that, since biotin is not required when oxalacetate is not decarboxylated, it acts on the decarboxylation of this compound. However, this is not regarded as proof of its action inasmuch as a somewhat artificial system is employed.

More direct evidence of the site of action of biotin in oxalacetate decarboxylation was obtained in the following manner. At pH 4, the spontaneous decomposition of oxalacetate is so great that the presence of enzymatic decarboxylation is difficult to demonstrate. At pH 6, however, the enzymatic decarboxylation is apparent, but at this pH pyruvate is rapidly decomposed by the cell suspensions, and semicarbazide cannot be used as an inhibitor because it combines with oxalacetic acid. As reported

TABLE IV

*Influence of Biotin upon Oxalacetate and Malate Decarboxylation*

(a) Oxalacetate decarboxylation, cells at pH 4 at 37° for 60 minutes, centrifuged, adjusted to pH 6.5, reaction run in 0.3 M phosphate, 896 microliters of added oxalacetate, 0.005 M NaCN, inorganic salt mixture, gas phase N<sub>2</sub>. Controls were boiled cells. (b) Malate decomposition, as in Table II, pH 4.

Experiment No	Cell treatment	Additions	Biotin, mγ per 3 ml	Oxalacetic acid Q <sub>CO<sub>2</sub></sub> (N)		Malate co (N)
				Observed	Less killed cells	
476	37°, pH 4, 60 min	None	0	362	225	187
		Vitamins*	20	600	463	350
		Biotin	100	545	408	350
	Heated, 100°, 10 min	Vitamins*	20	137		
478	37°, pH 4, 60 min	None	0	250	138	12
		Vitamins*	20	375	263	112
		“ less	0	204	92	12
		biotin†				
		Biotin	10	375	263	112
	Heated, 100°, 10 min	Vitamins	20	112		

\* Vitamins added per cup, 0.5 γ each of riboflavin, thiamine, pantothenic acid, nicotinic acid, folic acid, *p*-aminobenzoic acid, and 20 mγ of biotin.

† The same as above, biotin omitted.

by Kalnitsky and Werkman (8), 0.005 M NaCN largely inhibits the decomposition of pyruvate, while permitting oxalacetate decarboxylation. The cells were therefore rendered deficient by holding them at 37° at pH 4 for 60 minutes, whereupon they were centrifuged from the buffer, adjusted to pH 6.5, and tested for oxalacetate decarboxylase activity at pH 6 with boiled cells as a control. The data of Table IV illustrate that biotin is the active agent in the decarboxylation of oxalacetate.

However, there are suggestions in these data that the natural material derived from malate is not oxalacetic acid as such. These are similar to the indications reported by Kalnitsky and Werkman (8) and others (9, 10).

and are of two types. First, the rate of decomposition of added oxalacetate is relatively high, even at pH 6, especially when catalyzed by the killed cell substance. As is evident in Experiment 478 (Table IV), the malate system is capable of generating 112 microliters per hour per mg of cell nitrogen of a material yielding carbon dioxide. If this material was oxalacetate, it should decompose spontaneously, therefore, rates lower than 112 microliters (as in the "No additions" series) should not occur. That they do may be taken to indicate that the material originating from aspartic or malic acid is not oxalacetic acid as such. Even more striking results are obtained at pH 4, when oxalacetate is very rapidly decarboxylated. Experiments 409, 415, and 421 on aspartic acid (Table I) and Experiments 420, 424, and 444 on malic acid (Table II) should not be possible if the product were oxalacetate as such, since it would spontaneously decompose at pH 4 at many times the rates recorded. Second, malate or aspartate liberates carbon dioxide in the presence of semicarbazide (pH 4) which, however, unites readily with added oxalacetate and prevents its decarboxylation. The product derived from malate or aspartate appears to be one which does not combine with semicarbazide, a property of oxalacetate, therefore, it is not oxalacetate as such. That the semicarbazide penetrates the cells may be seen from the fact that it combines with the pyruvate formed and prevents its decomposition. In common with previous workers who have recorded this phenomenon, we have so far been unable to proceed further experimentally. Since adenosine triphosphate is required for the Wood-Werkman reaction (11, 12), a phosphorylated derivative of oxalacetate may be the natural substrate, derived from malate, for whose decarboxylation biotin is required. In our experiments, the production of carbon dioxide from malate has not been possible in the absence of phosphate.

#### SUMMARY

By a process of holding cells of *Escherichia coli* at pH 4 at 37° for 15 to 60 minutes, their ability to produce carbon dioxide from aspartic, malic, or oxalacetic acid is decreased. This activity is restored upon the addition of biotin. From the data cited, the action of biotin is localized in oxalacetate decarboxylase, suggesting that it functions as the coenzyme. Although the substance derived from aspartic and malic acids may not be oxalacetic acid as such, biotin is also required for its decarboxylation.

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# NATURALLY OCCURRING GLYCEROL ETHERS

## III SELACHYL ALCOHOL AND ITS GEOMETRICAL ISOMER

By ERICH BALR AND HERMANN O L FISCHER

WITH A SECTION IN THE TEXT BY LEON J RUBIN

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(Received for publication, June 12, 1947)

Selachyl alcohol is D- $\alpha$ -oleyl glycerol ether<sup>1</sup>. The present authors were able to prove its configuration in 1941 (1), and attempted its synthesis in 1944, in cooperation with Leon J Rubin (2). In analogy to the syntheses of butyl alcohol and chmyl alcohol (1), the sodium salt of L(-)-acetone glycerol was combined with oleyl-*p*-toluenesulfonate. The condensation product, after deacetonation, yielded a crystalline substance  $C_{21}H_{42}O_3$  which showed the rotation of natural selachyl alcohol and a melting point of 48.5-49.5°. Since the melting point of the "synthetic product" was higher than any observed on natural selachyl alcohol, it was thought possible that elaidinization had occurred during synthesis. Oleyl alcohol was subjected to the conditions of the synthesis and recovered essentially unchanged. It was known that sodium alcoholates do not bring about elaidinization (3). The authors therefore believed at the time that their synthetic product of the melting point 48.5-49.5° was a derivative of oleyl alcohol. The difference in the melting points of the "synthetic product" and the natural selachyl alcohol remained unexplained.

New doubts as to the oleyl structure of our synthetic compound arose later when Rubin heated natural selachyl alcohol with selenium and succeeded in transforming it into a compound with an approximate melting point of 48-49°. Furthermore, the condensation of elaidyl *p*-toluenesulfonate with the sodium salt of L(-)-acetone glycerol yielded also a compound with a melting point of 50-51°. These observations prompted us to request Professor M F Crawford, of this University, to study the infra-red and Raman spectra of the compound melting at 48.5-49.5°. The results of this investigation proved unequivocally that this compound is the trans derivative.

Obviously a new synthesis for the D- $\alpha$ -oleyl glycerol ether was required. Since oleyl alcohol, in contrast to its toluenesulfonate, was not elaidinized by sodium alcoholates (2), the original procedure was modified by condensing the sodium salt of oleyl alcohol with  $\alpha$ -(*p*-toluenesulfonyl)L-acetone glycerol. Hydrolysis of the condensation product with acetic

<sup>1</sup> A review of the earlier literature can be found in Paper II of this series (2).

acid and subsequent treatment of the hydrolysis product with potassium hydroxide solution yielded pure D- $\alpha$ -oleyl glycerol ether (m p 17.6–19.0° and  $[\alpha]_D^{23} = -4.5^\circ$ ). Our purest sample of natural D-selachyl alcohol showed the same melting point and rotation (for the rotation cf. also Toyama (4)). The condensation, however, yielded the D- $\alpha$ -oleyl glycerol ether in a yield of 16 per cent only. This modification of the original procedure was suggested by Leon J. Rubin, who established its feasibility by synthesizing DL-chimyl alcohol and DL-selachyl alcohol in this manner. Professor Crawford, who again was kind enough to investigate the infra-red spectrum of the natural selachyl alcohol and the new synthetic product, states "The synthetic compound (m p 17–19°) and natural selachyl alcohol absorb strongly at  $1652\text{ cm}^{-1}$ , the C=C characteristic frequency, whereas the product (m p 48.5–49.5°) of the first synthetic procedure (2) and the compound (m p 48–49°) formed by elaidinization of natural selachyl alcohol absorb very weakly at this frequency. Since the trans form can be considered to have a center of symmetry so far as the C=C localized vibration is concerned, the change in the dipole moment associated with this vibration will be zero, consequently, the trans form should have negligible absorption at  $1652\text{ cm}^{-1}$ . Thus the two compounds with m p 17–19° are cis and those with m p 48–49° are trans."

The infra-red and Raman spectra of the natural selachyl alcohol and the synthetic oleyl and elaidyl glycerols will be reported by Professor M. F. Crawford and Dr. L. J. Rubin in detail elsewhere.

In view of the new experimental evidence brought forward, we do not hesitate to state that the new procedure produces, though in poor yields, D- $\alpha$ -oleyl glycerol ether (m p 17.6–19.0°,  $[\alpha]_D^{23} = -4.5^\circ$ ) which is identical with selachyl alcohol from natural sources.

#### EXPERIMENTAL

##### *D-Selachyl Alcohol*

*$\alpha$ -(p-Toluenesulfonyl)L-acetone Glycerol*—To a gently agitated and cooled mixture of 19.0 gm. of *p*-toluenesulfonyl chloride and 10 cc. of dry pyridine were added 13.2 gm. of freshly prepared L(-)-acetone glycerol ( $[\alpha]_D = -13.9^\circ$ ) (5). The reaction mixture was kept in an ice bath until the reaction had subsided (approximately 1 hour). After standing 48 hours at room temperature, a crystalline sludge had formed which was poured with stirring into 500 cc. of ice water. The supernatant liquid was immediately decanted and the heavy oil, dissolved in ether, washed with sodium carbonate solution. The ether solution was dried with anhydrous sodium sulfate and concentrated *in vacuo* to a thick syrup. The residue (24.2 gm.) was dissolved in a mixture of 150 cc. of dry ether and 300 cc. of petroleum ether (b p 30–60°), the solution cooled to  $-70^\circ$ , and the thick sludge filtered with suction on a cooled Buchner funnel. The substance, which



liquefies at room temperature, was freed *in vacuo* from adhering solvent. The yield of  $\alpha$ -(*p*-toluenesulfonyl)-L-acetone glycerol was 23.0 gm (80.4 per cent).  $n_D^{22} = 1.5051$ ,  $d_4^{24} = 1.208$ ,  $[\alpha]_D^{24} = +6.7^\circ$  (in substance),  $[\alpha]_D^{24} = +1.6^\circ$  (c 13, dry ethanol).

<i>Analysis</i> — $C_{13}H_{15}O_5S$	Calculated	C 54.51, H 6.29, acetone 20.3
286.2	Found	" 54.60, " 6.28, " 20.6, 20.0

*Acetone Compound of D-Sclachyl Alcohol*—190 cc of a molar solution of sodium naphthalene in glycol dimethyl ether were prepared according to the directions of Scott, Walker, and Hansley (6). Into the cooled solution was run with stirring a solution of 50.2 gm of oleyl alcohol (2)<sup>2</sup> in 50 cc of glycol dimethyl ether, and the stirring was continued until the formation of the sodium compound of oleyl alcohol was completed. A solution of 53.5 gm of  $\alpha$ -(*p*-toluenesulfonyl)-L-acetone glycerol in 100 cc of glycol dimethyl ether was then added and the mixture refluxed gently in an atmosphere of dry nitrogen for a period of 44 hours. The sodium *p*-toluenesulfonate was filtered off with suction, the solid washed with ether, and the combined filtrates concentrated under diminished pressure. In order to remove most of the naphthalene and dihydronaphthalene, the residue was heated gradually in a vacuum of 10 to 15 mm to a temperature of  $160^\circ$  (bath). The remaining oil was taken up in ether, the solution washed several times with water, dried with anhydrous sodium sulfate, and again concentrated. The residue was distilled in a molecular still (7) at a pressure of  $2 \times 10^{-2}$  mm. Three fractions were collected. Fraction A, temperature of oil bath  $130$ – $150^\circ$ , speed of distillation 12 to 6 drops per minute,  $n_D^{26} = 1.4620$ , Fraction B, temperature of oil bath  $150$ – $160^\circ$ , speed of distillation 15 to 4 drops per minute, 20 gm,  $n_D^{27} = 1.4640$ , 7.5 per cent acetone, Fraction C, temperature of oil bath  $160$ – $170^\circ$ , speed of distillation 4 to 0.2 drops per minute, 16.3 gm,  $n_D^{28} = 1.4585$ , 13.3 per cent acetone,  $[\alpha]_D = -11.6^\circ$ , in substance. A considerable amount of substance (18.9 gm) containing not more than 0.5 per cent of acetone remained undistilled,  $n_D^{28} = 1.4710$ .

Fraction C, containing approximately 88 per cent of the desired acetone compound, was again fractionally distilled (molecular still) at a pressure of  $1 \times 10^{-3}$  to  $5 \times 10^{-4}$  mm and a speed of 1 drop of distillate per minute. Two fractions were collected: (a)  $95$ – $100^\circ$  (air bath), 4.0 gm,  $n_D^{26} = 1.4637$ ,  $[\alpha]_D = -9.44^\circ$ , (b)  $100$ – $115^\circ$  (air bath), 11.4 gm (15.9 per cent yield),  $n_D^{26} = 1.4568$ ,  $d_4^{22} = 0.898$ ,  $[\alpha]_D^{26} = -12.97^\circ$ , in substance.

<i>Analysis</i> — $C_{24}H_{36}O_3$ (382.4)	Calculated	C 75.31, H 12.09, acetone 15.18
	Found	" 75.56, " 12.35, " 14.92
		75.58 12.40

<sup>2</sup> This and all following operations were carried out in an atmosphere of nitrogen whenever possible.

acid and subsequent treatment of the hydrolysis product with potassium hydroxide solution yielded pure D- $\alpha$ -oleyl glycerol ether (m p 17.6–19.0° and  $[\alpha]_D^{23} = -4.5^\circ$ ). Our purest sample of natural D-selachyl alcohol showed the same melting point and rotation (for the rotation cf also Toyama (4)). The condensation, however, yielded the D- $\alpha$ -oleyl glycerol ether in a yield of 16 per cent only. This modification of the original procedure was suggested by Leon J. Rubin, who established its feasibility by synthesizing DL-chimyl alcohol and DL-selachyl alcohol in this manner. Professor Crawford, who again was kind enough to investigate the infra-red spectrum of the natural selachyl alcohol and the new synthetic product, states "The synthetic compound (m p 17–19°) and natural selachyl alcohol absorb strongly at 1652 cm<sup>-1</sup>, the C=C characteristic frequency, whereas the product (m p 48.5–49.5°) of the first synthetic procedure (2) and the compound (m p 48–49°) formed by elaidinization of natural selachyl alcohol absorb very weakly at this frequency. Since the trans form can be considered to have a center of symmetry so far as the C=C localized vibration is concerned, the change in the dipole moment associated with this vibration will be zero, consequently, the trans form should have negligible absorption at 1652 cm<sup>-1</sup>. Thus the two compounds with m p 17–19° are cis and those with m p 48–49° are trans."

The infra-red and Raman spectra of the natural selachyl alcohol and the synthetic oleyl and elaidyl glycerols will be reported by Professor M. F. Crawford and Dr. L. J. Rubin in detail elsewhere.

In view of the new experimental evidence brought forward, we do not hesitate to state that the new procedure produces, though in poor yields, D- $\alpha$ -oleyl glycerol ether (m p 17.6–19.0°,  $[\alpha]_D^{23} = -4.5^\circ$ ) which is identical with selachyl alcohol from natural sources.

## EXPERIMENTAL

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mixture was gently refluxed for 2½ hours. The sodium *p*-toluenesulfonate was centrifuged off, washed several times with ether, and the combined supernatant liquids concentrated at reduced pressure. The residue was gradually heated to a temperature of 160° in a vacuum of 10 to 15 mm. The remaining oil was taken up in ether, the solution washed rapidly with ice-cold water, dried with sodium sulfate, and again concentrated. The oil was fractionally distilled in a molecular still (7) at a pressure of  $3 \times 10^{-3}$  mm while the temperature of the air bath was gradually raised from 95–105° (speed of distillation 1 drop or less per minute). Two fractions were collected. The first, weighing 1.3 gm ( $n_D^{25} = 1.4550$ ) contained all the impurities. The second fraction, weighing 2.9 gm (49.3 per cent,  $n_D^{25} = 1.4559$ ) was the pure acetone compound of D- $\alpha$ -elaidyl glycerol.

Analysis—C <sub>44</sub> H <sub>80</sub> O <sub>3</sub> (382.4)	Calculated	C 75.31, H 12.09
	Found	" 75.20, " 12.02

*D- $\alpha$ -Elaidyl Glycerol Ether*—2.9 gm of the acetone compound of D- $\alpha$ -elaidyl glycerol were dissolved in 25 cc of 80 per cent acetic acid at 80° and the solution kept at the same temperature for 2 hours. On the addition of 100 cc of cold water a semisolid material precipitated which was sucked off and triturated on the steam bath with a slight excess of dilute potassium hydroxide solution for 30 minutes. The product was taken up in ether, and the solution washed with water until free from alkali and dried with anhydrous sodium sulfate. The ether solution was evaporated to dryness and the residue (2.25 gm, m.p. 48–49°) recrystallized from 10 cc of dry acetone at –15°. 2.01 gm of pure D- $\alpha$ -elaidyl glycerol were obtained, melting at from 50.0–51.0° (corrected). The mixed melting point of the D- $\alpha$ -elaidyl glycerol with a sample of the "selachyl alcohol (m.p. 48.5–49.5°)," which was prepared by condensation of the oleyl-*p*-toluenesulfonate with the sodium salt of L(-)-acetone glycerol and subsequent acid hydrolysis (2), showed no depression.

Analysis—C <sub>21</sub> H <sub>42</sub> O <sub>3</sub> (342.6)	Calculated	C 73.60, H 12.32
	Found	" 73.78 " 12.47

### *Elaidinization of Natural Selachyl Alcohol with Selenium*

By LEON J. RUBIN

5 gm of purified selachyl alcohol<sup>4</sup> from dogfish liver oil ( $n_D^{24} = 1.4680$ ) were heated in a Pyrex test-tube with 15 mg of finely powdered amorphous

<sup>4</sup> The unsaponifiable matter of dogfish liver oil was dissolved in acetone, filtered with suction, and the filtrate evaporated. The residue was dissolved in methanol, again chilled, and filtered. This procedure removed most of the saponifiable material and the sterols. The residue of the methanol solution was acetylated, the mixture of acetates fractionally distilled *in vacuo*. Saponification of the fractions of the diacetyl selachyl alcohol gave the selachyl alcohol used for elaidinization.

selenium at 220° for a period of 2 hours. A stream of pure nitrogen<sup>5</sup> was passed through the reaction mixture to exclude air and provide agitation. The warm oil was dissolved in 20 cc of acetone and the material allowed to crystallize in the refrigerator. A crystalline product (2.3 gm) was obtained which melted at from 44–45°. On recrystallizing twice from acetone and once from petroleum ether (b.p. 60–80°), the melting point was raised to 47–48°.

<i>Analysis</i> — $C_{21}H_{42}O_3$	Calculated	C 73.60, H 12.32, I No 74.0
	Found	" 73.64, " 12.48, " " 71.4
		73.72 12.38

#### SUMMARY

*n*- $\alpha$ -Oleyl glycerol ether (m.p. 17–19°), identical with natural selachyl alcohol, and *n*- $\alpha$ -elaidyl glycerol ether (m.p. 48–49°), the trans isomer, have been synthesized. The geometrical configurations were established by examination of their infra-red absorption spectra.

We wish to express our sincere appreciation to Professor M. F. Crawford, University of Toronto, for his friendly cooperation, which was decisive in elucidating the cis-trans isomerism of the natural selachyl alcohol and the two synthetic products, to Professor R. G. Sinclair, Queen's University, Kingston, Ontario, for a sample of elaidic acid methyl ester, to Dr. K. C. D. Hickman, Distillation Products, Inc., Rochester, New York, for a generous supply of unsaponifiable matter of dogfish liver oil, and to Dr. Leon J. Rubin for conducting the preliminary experiments.

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<sup>5</sup> Tank nitrogen was purified and dried by passing it in succession through Fieser's solution (8), lead acetate solution, concentrated sulfuric acid, and drierite.

# A MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF MANGANESE\*

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(Received for publication, June 2, 1947)

A salt mixture containing manganese has been widely used in media for lactic acid bacteria (1-3). The growth-enhancing properties of manganese for lactic acid bacteria has been noted by various investigators (4, 5), and Moeller (6), using a highly purified medium of synthetic nature, found it essential for a strain of *Streptobacterium plantarum*. More recently MacLeod and Snell (7) have shown that manganese is essential for many organisms of this group.

The successful use of lactic acid bacteria for assay of vitamins and amino acids suggested study of their applicability to manganese assay. Although many chemical methods for the estimation of this ion in a host of different materials have appeared, application of many of these to biological materials, in which only a few micrograms of manganese may be present with large quantities of interfering materials (e.g. iron in blood), has not been generally satisfactory. Development and use of a microbiological method will be described below.

## EXPERIMENTAL

**Organism**—Preliminary work was done with *Lactobacillus casei*. *Lactobacillus arabinosus* 17-5 was soon found to respond more favorably to manganese addition, and was used in most of the work reported.

**Stock Cultures and Inocula**—Stock cultures were carried by semimonthly transfer in the medium recommended by Nymon and Goitner (8). This contains tryptone,  $K_2HPO_4$ , glucose, liver extract, agar, and water. The stab cultures were incubated for 48 hours at 37° and then stored in a refrigerator until used. This same medium, with agar omitted, was used to prepare the inoculum. For this purpose, a transfer was made from a stab culture to a tube (10 cc) of sterile liquid medium which was incubated for 24 hours at 37°. The cells were then centrifuged, the supernatant liquid poured off and replaced with 10 cc of sterile saline solution, and the cells resuspended by carefully swirling the tubes. 1 drop of this suspension was used to inoculate each assay tube.

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Tennessee Eastman Corporation of Kingsport, Tennessee.

*Medium*—The medium of Roberts and Snell (9), with manganese omitted, was used with minor modifications. Its composition is given in Table I. A major obstacle in using such a medium for manganese assay was the fact that, despite use of ingredients of the highest obtainable purity, sufficient manganese was always present in the final medium to permit near maximal growth and acid production of the test organisms.

It was found, however, that the test organisms themselves could be used as a means of removing this manganese from the medium. For this pur-

TABLE I  
*Composition of Basal Medium*

Constituent	Amount per liter of double strength medium	Constituent	Amount per liter of double strength medium
Enzymatic casein digest*	800 cc	Guanine	20 mg
Glucose	40 gm	Adenine sulfate	20 "
Sodium acetate	20 "	Calcium pantothenate	10 "
K <sub>2</sub> HPO <sub>4</sub>	0.5 "	Nicotinic acid	10 "
KH <sub>2</sub> PO <sub>4</sub>	0.5 "	Pyridoxamine	10 "
Salt mixture†	40 cc	Riboflavin	10 "
Water	160 "	Thiamine	10 "
Cystine	400 mg	Biotin	250 γ
Uracil	20 "	Folic acid	250 "

\* The enzymatic digest was prepared by suspending 120 gm of Labco casein in 2 liters of 0.8 per cent sodium bicarbonate solution. 600 mg of pancreatin in 15 to 20 cc of water were added to the suspension and mixed to insure uniform distribution. A thin layer of toluene was spread over the top and the mixture placed in an incubator at 37° for 48 hours. After incubation the mixture was steamed for 30 minutes and the bicarbonate was neutralized with 7 cc of glacial acetic acid. The material was filtered by the use of Super-Cel under medium suction. The volume was then adjusted to 2400 cc and stored under toluene at room temperature until used. 1 cc of this solution was equivalent to 50 mg of casein.

† The salt mixture contained the following: 10 gm of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.5 gm of NaCl, and 0.5 gm of FeSO<sub>4</sub> · 7H<sub>2</sub>O in 250 cc of water. All salts were of c. p. reagent quality.

pose, a liter of double strength medium (Table I) was prepared and sterilized by autoclaving at 15 pounds pressure for 5 minutes. After cooling, this was inoculated with 20 cc of the inoculum suspension and incubated 24 hours at 37°. The cells were then centrifuged and the pH was adjusted to 6.8 to 7.0 with NH<sub>4</sub>OH. During this short pretreatment, 1 to 2 cc of 0.1 N acid per 5 cc of double strength medium were usually produced. An additional allotment of uracil, guanine, adenine, *p*-aminobenzoic acid, and the vitamins was then added to the medium in the amounts shown in Table I. The medium was then ready for use in assays.

The effectiveness of this treatment in removing manganese present as a contaminant in the basal medium is shown by the data in Table II. When *Lactobacillus casei* was used as the test organism instead of *Lactobacillus arabinosus*, the same general picture was obtained. *L. casei*, however, seemed to be considerably more sensitive to traces of manganese than did *L. arabinosus*, since "blanks" of 5 to 6 cc of 0.1 N acid were often obtained even with the pretreated medium. It was possible, by using *L. casei* for pretreating the medium and by carrying through this procedure two or more times, to reduce the manganese content to a level which did not support appreciable growth of this organism in the absence of added manganese. The inconvenience of such repeated treatments led to the abandonment of *L. casei* as a test organism in favor of *L. arabinosus*.

*Assay Procedure*—The usual microbiological assay techniques were used throughout this study. Briefly the procedure was as follows. The final

TABLE II

*Effect of Pretreatment of Medium on Acid Production by Lactobacillus arabinosus in Presence and Absence of Added Manganese*

Treatment of medium	0.1 N acid produced per 10 cc	
	No added Mn (blanks)	Excess Mn (100 $\gamma$ per 10 cc)
	cc	cc
None	8.35	17.7
Pretreated with <i>L. arabinosus</i>	1.5	15.7

volume in the assay tubes (18  $\times$  150 mm Pyrex culture tubes) was made by diluting 5 cc of the pretreated double strength medium to 10 cc with either water, sample, or water plus sample. Each sample aliquot was assayed in duplicate. Four to six aliquots of varying size were taken for each sample. All tubes and contents were sterilized by autoclaving 15 minutes at 15 pounds pressure. The tubes were cooled and inoculated, then incubated at 37° for 72 hours.

Response to added manganese was measured by electrometric titration of the acid produced. For this purpose, a previously described calomel half-cell-quinhydrone system with a sensitive galvanometer was found convenient. Since certain samples were turbid, the turbidimetric method of assay was never used. In practice, titration values were considered checks if within 0.3 cc of alkali on duplicate tubes.

*Standard Curve*—A standard solution was prepared by dissolving sufficient  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  in 1 liter of water to give a final concentration of 0.5  $\gamma$  of manganese per cc. At this concentration, the smallest volume which must be pipetted in preparing a standard curve is 0.1 cc. A typical standard

curve, showing response to added manganese and the assay range used, is shown in Fig 1. The response of *Lactobacillus arabinosus* to manganese added in a solution of  $\text{KMnO}_4$  is shown in Experiment 2 of Fig 1. It is seen that the curve was similar to the standard curve obtained during the same assay. It was concluded, then, that ionic valency would not present a problem either in sample preparation or as a source of error in the quantitative determination of manganese. Undoubtedly the presence of many oxidizable materials in this medium and the lowering of the pH of the medium during bacterial growth would both tend to maintain manganese in the lower valence state.

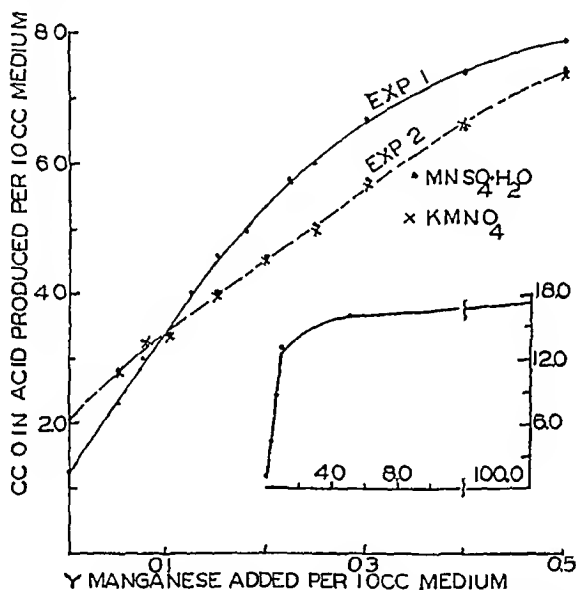


FIG 1 The response of *Lactobacillus arabinosus* to added manganese in a manganese deficient medium. The insert shows the effect of high concentrations of manganese.

It can be seen in the insert (Fig 1) that acid production continues to increase with manganese additions until titration values of 15 to 17 cc of 0.1 N acid per 10 cc of medium were reached. At levels higher than 0.5 γ of manganese ion per 10 cc, however, the slope of the curve fell off rapidly and was not of great value for assay purposes. By comparing many assays it was found that the most accurate and satisfactory results were obtained when the portion of the standard curve between 0.1 and 0.3 γ of manganese was used. Values obtained above and below this segment were not necessarily inaccurate but tended to be more erratic. To obtain the manganese content of a sample, results calculated from at least three sample aliquots



promote considerable growth. If the amount present is not too great, it is often possible, merely by growing in the medium an organism requiring the ion for growth, to reduce the concentration of the ion in the medium to a level which will not support growth of the organism. This procedure for removing traces of inorganic ions is referred to throughout this paper as "pretreatment" of the medium. It is carried out as follows.

1 liter of the double strength medium, complete except for its complement of the inorganic ion under investigation, is autoclaved 5 to 10 minutes at 15 pounds pressure. The time of autoclaving is reduced to a minimum to prevent excessive caramelization, should repeated pretreatment be necessary. After cooling, the sterilized medium is inoculated with 10 cc of the inoculum culture, then incubated for 24 hours at the appropriate temperature. The cells are removed by centrifugation. For this purpose, plastic (Iustron) centrifuge tubes and a centrifuge equipped with a conical head are very satisfactory. After centrifugation, the clear medium is decanted from the cells and pooled. The pH is readjusted to 6.5 with a solution of ammonia prepared by boiling commercial ammonium hydroxide and redissolving the evolved ammonia in distilled water. The medium thus pretreated may be stored under toluene in the cold room until use. Since the concentrations of certain essential ingredients of the medium (other than the essential mineral element) may be reduced to levels below those required for optimal growth, the medium is "fortified" just before use by adding amounts of glucose, vitamins, and purine bases equal to those used in the original medium.

*Test Procedure*—The customary techniques of microbiological assay were used (*cf* (12)). 5 cc of the pretreated double strength medium were added to each of a series of 18 × 150 mm Pyrex test-tubes. Appropriate quantities of solutions of the inorganic ion to be investigated were then added, and the total volume of each tube adjusted to 10 cc with distilled water. In all cases,  $Mn^{++}$  was added as  $MnSO_4 \cdot H_2O$ ,  $Mg^{++}$  as  $MgSO_4 \cdot 7H_2O$ ,  $Fe^{++}$  as  $FeSO_4 \cdot 7H_2O$ ,  $K^+$  as KCl, and  $PO_4^{--}$  as  $KH_2PO_4$ . All salts were of reagent quality. Additions were made to supply the amounts of the ions indicated, not the salts. All tubes were covered with aluminum caps, autoclaved 10 minutes at 15 pounds pressure, cooled, and inoculated. For inoculum, the inoculum culture (10 cc) was centrifuged, the supernatant medium removed, and the cells resuspended in sterile 0.9 per cent NaCl solution. This suspension was again centrifuged, the supernatant liquid discarded, and the cells resuspended as before in 10 cc of sterile saline. When a "heavy" inoculum was desired, 1 drop of this suspension was added to each culture tube, if a "light" inoculum was needed, 1 cc of this suspension was added to 100 cc of sterile saline and 1 drop of this suspension added to each culture tube. After the growth period (usually 24 hours) the turbidity of each

## EXPERIMENTAL

*Cultures and Inocula*—Cultures used were *Lactobacillus arabinosus* 17-5, *Lactobacillus casei*, *Lactobacillus delbrueckii* LD-3, *Lactobacillus fermenti* 36, *Lactobacillus pentosus* 124-2, *Leuconostoc mesenteroides* P-60 and 9135, and *Streptococcus faecalis* R. Cultures were carried as stabs in yeast-dextrose agar. Inoculum cultures were incubated 16 to 18 hours in yeast extract-glucose broth (1 per cent Difco yeast extract and 1 per cent glucose). On some occasions, the inoculum may be grown in the mineral-deficient

TABLE I  
Composition of Basal Medium\*

	Amounts per 10 cc final medium			Amounts per 10 cc final medium	
1 Casein (enzymatic hydrolysate)	100	mg	13 Riboflavin	2	γ
2 Asparagine	1	"	14 Niacin	2	"
3 DL-Tryptophan	0.5	"	15 p-Aminobenzoic acid	1	"
4 Cystine	1	"	16 Folic acid	0.05	"
5 Adenine HCl	0.1	"	17 Biotin	0.01	"
6 Guanine "	0.1	"	18 KH <sub>2</sub> PO <sub>4</sub>	10	mg
7 Uracil	0.1	"	19 K <sub>2</sub> HPO <sub>4</sub>	10	"
8 Glucose	100	"	20 MgSO <sub>4</sub> 7H <sub>2</sub> O	2	"
9 Sodium acetate	60	"	21 NaCl	0.1	"
10 Pyridoxal HCl	1	γ	22 FeSO <sub>4</sub> 7H <sub>2</sub> O	0.1	"
11 Thiamine "	1	"	23 MnSO <sub>4</sub> H <sub>2</sub> O	0.1	"
12 Calcium pantothenate	2	"			

\* For convenience separate solutions were prepared of (a) purines and pyrimidines (Constituents 5 to 7), (b) the vitamins (Constituents 10 to 17), (c) Salts A (Constituents 18 and 19), and (d) Salts B (Constituents 20 to 23). Salts A and Salts B constitute Speakman's salt mixture. Constituents 1 to 7 plus Salts A were combined to form a single stock solution, to which glucose, acetate, the vitamin solution, and the appropriate salts were added to form the final medium.

medium. Cultures of *Leuconostoc mesenteroides* and *Lactobacillus pentosus* were incubated at 30°, other cultures were incubated at 37°.

*Basal Medium*—The medium used (Table I) was a slight modification of that reported by Roberts and Snell (12). The enzymatic digest of casein was prepared as described previously (12) with the exception that hydrochloric acid was used to neutralize the digest and all charcoal treatments were omitted. The single mineral element to be studied was omitted from the medium in each case.

*Preparation of Mineral-Deficient Medium*—Despite omission of the inorganic ion under study, sufficient is usually present as a contaminant to

promote considerable growth. If the amount present is not too great, it is often possible, merely by growing in the medium an organism requiring the ion for growth, to reduce the concentration of the ion in the medium to a level which will not support growth of the organism. This procedure for removing traces of inorganic ions is referred to throughout this paper as "pretreatment" of the medium. It is carried out as follows.

1 liter of the double strength medium, complete except for its complement of the inorganic ion under investigation, is autoclaved 5 to 10 minutes at 15 pounds pressure. The time of autoclaving is reduced to a minimum to prevent excessive caramelization, should repeated pretreatment be necessary. After cooling, the sterilized medium is inoculated with 10 cc of the inoculum culture, then incubated for 24 hours at the appropriate temperature. The cells are removed by centrifugation. For this purpose, plastic (Iustron) centrifuge tubes and a centrifuge equipped with a conical head are very satisfactory. After centrifugation, the clear medium is decanted from the cells and pooled. The pH is readjusted to 6.5 with a solution of ammonia prepared by boiling commercial ammonium hydroxide and redissolving the evolved ammonia in distilled water. The medium thus pretreated may be stored under toluene in the cold room until use. Since the concentrations of certain essential ingredients of the medium (other than the essential mineral element) may be reduced to levels below those required for optimal growth, the medium is "fortified" just before use by adding amounts of glucose, vitamins, and purine bases equal to those used in the original medium.

*Test Procedure*—The customary techniques of microbiological assay were used (*cf* (12)). 5 cc of the pretreated double strength medium were added to each of a series of 18 × 150 mm Pyrex test-tubes. Appropriate quantities of solutions of the inorganic ion to be investigated were then added, and the total volume of each tube adjusted to 10 cc with distilled water. In all cases,  $Mn^{++}$  was added as  $MnSO_4 \cdot H_2O$ ,  $Mg^{++}$  as  $MgSO_4 \cdot 7H_2O$ ,  $Fe^{++}$  as  $FeSO_4 \cdot 7H_2O$ ,  $K^+$  as KCl, and  $PO_4^{--}$  as  $KH_2PO_4$ . All salts were of reagent quality. Additions were made to supply the amounts of the ions indicated, not the salts. All tubes were covered with aluminum caps, autoclaved 10 minutes at 15 pounds pressure, cooled, and inoculated. For inoculum, the inoculum culture (10 cc) was centrifuged, the supernatant medium removed, and the cells resuspended in sterile 0.9 per cent NaCl solution. This suspension was again centrifuged, the supernatant liquid discarded, and the cells resuspended as before in 10 cc of sterile saline. When a "heavy" inoculum was desired, 1 drop of this suspension was added to each culture tube, if a "light" inoculum was needed, 1 cc of this suspension was added to 100 cc of sterile saline and 1 drop of this suspension added to each culture tube. After the growth period (usually 24 hours) the turbidity of each

culture was determined in the Evelyn colorimeter, with the 660 m $\mu$  filter and the 10 cc aperture

**Manganese**—The manganese requirements of eight lactic acid bacteria were determined in a medium which had been pretreated once with *Lactobacillus arabinosus*. The results are given in Table II. With the exception of *Lactobacillus casei* and *Streptococcus faecalis*, none of the organisms grew appreciably unless Mn<sup>++</sup> was added. Maximum growth in 24 hours was achieved with considerably less than 100  $\gamma$  of Mn<sup>++</sup> per 10 cc of medium.

TABLE II  
Response of Lactic Acid Bacteria to Added Manganese on Manganese-Deficient Medium\*

	Mn <sup>++</sup> per 10 cc				
	0 $\gamma$	0.1 $\gamma$	0.3 $\gamma$	1.0 $\gamma$	100 $\gamma$
	Galvanometer readings†				
<i>Lactobacillus arabinosus</i>	98	80	61	37	28
" <i>delbrueckii</i>	85	58	47	33	27
" <i>casei</i>	73	43	30	25	25
<i>Streptococcus faecalis</i>	66	62	59	58	54
<i>Leuconostoc mesenteroides</i> (P-60)	98	84	71	49	42
" " (9135)	95	81	72	63	63
<i>Lactobacillus pentosus</i>	97	71	51	38	38
" <i>fermentis</i>	95	87	80	63	34
" <i>casei</i> †	93	69	54	40	30

\* The basal medium, with manganese omitted, was pretreated once with *Lactobacillus arabinosus*.

† The galvanometer readings (Evelyn colorimeter) represent per cent of the incident light transmitted. The uninoculated medium was set to read 100.

‡ In this instance, the basal medium, with manganese omitted, was pretreated twice with *Lactobacillus casei*.

*L. casei* and *Streptococcus faecalis* grew appreciably without added manganese, growth was greatly enhanced, however, by its addition. It is possible either that the organisms do not require manganese and are stimulated by it, or their manganese requirements are low and sufficient iron remains in the medium after pretreatment with *L. arabinosus* to permit their limited growth.

To decide between these possibilities, a portion of the basal medium was pretreated twice in succession with *Lactobacillus casei*. Inoculum for the second pretreatment was washed with saline to avoid introduction of manganese from this source. The response of *L. casei* to added manganese in the twice pretreated medium is also shown in Table II. Growth in the absence of manganese was slight, good growth occurred when manganese

was added. It is concluded, therefore, that *L. casei* also requires manganese for growth. Even on media similar to this, however, it was not possible to demonstrate that *Streptococcus faecalis* required manganese for growth.

The specificity of the requirement for manganese was investigated to a limited extent. Reagent grade salts of  $Mg^{++}$ ,  $Co^{++}$ ,  $Ni^{++}$ , and  $Ca^{++}$ , when tested at a concentration of 1000  $\gamma$  of the metallic ion per 10 cc, showed slight growth-promoting effects. After recrystallization from distilled water, however, they were inactive. Their initial activity was thus due to contamination with manganese. Ferrous salts showed low activity (about 0.08 per cent that of manganese) which was not altered by reprecipitation from dilute sulfuric acid solution with ethanol. Ferrous ion prepared by dissolving analytical grade iron wire in dilute hydrochloric acid showed similar activity. Although the activity of ferrous ion is believed to result from contamination, no positive data are available to prove this point.

**Magnesium**—Preliminary growth trials on a medium to which no magnesium was added showed that, of the eight cultures tested, only *Lactobacillus arabinosus* and *L. casei* were significantly stimulated by addition of magnesium. Three successive pretreatments of the deficient medium with *L. casei* failed to reduce growth in the unsupplemented medium. It was decided, therefore, to pretreat the medium with yeast, which is known to require magnesium for growth. For this purpose, the magnesium-deficient basal medium was supplemented with  $CaCl_2$  (1 mg per cc) and inositol (50  $\gamma$  per cc), adjusted to pH 5.5, dispensed in 100 cc quantities into 500 cc Erlenmeyer flasks, and autoclaved for 5 minutes. After cooling, it was inoculated with a suspension of *Saccharomyces carlsbergensis* 4228, and incubated with shaking for 24 hours at 30°. The heavy growth which resulted was removed by centrifugation and the pretreatment repeated twice. The ability of this pretreated medium to support growth of *S. carlsbergensis* and of *L. casei* is shown in Table III. It is evident that the medium was deficient in magnesium for yeast, since it supported only slight growth of this organism unless magnesium was added. The medium supported good growth of *L. casei* in the absence of added magnesium, although magnesium does stimulate growth considerably.

No definite conclusions concerning the magnesium requirements of *Lactobacillus casei* can be drawn from these data, except that this requirement, if it exists, is of a lower order of magnitude than that of yeast. If the amount required is sufficiently small compared to that initially present as a contaminant, even repeated pretreatments will fail to show up the requirement. Similarly, contamination from glassware, etc., may be sufficient to supply the amounts required, and this source of essential ions could not be removed by the procedures used here. Further evidence bearing on the magnesium requirements of these organisms will be discussed later.

*Potassium*—To prepare a potassium-deficient medium, the potassium phosphates of the basal medium were replaced by equimolar amounts of sodium phosphates, and the medium was pretreated once with *Lactobacillus arabinosus*. It is evident from Table IV that all of the organisms tested require potassium for growth.

TABLE III  
Response of *Lactobacillus casei* and *Saccharomyces carlsbergensis* to Magnesium in Magnesium-Deficient Medium\*

	Mg <sup>++</sup> per 10 cc				
	0 γ	0.1 γ	1 γ	100 γ	1000 γ
	Galvanometer readings				
<i>S. carlsbergensis</i>	86				19
<i>L. casei</i>	41	35	34	28	27

\* The basal medium with magnesium omitted was pretreated three successive times with *Saccharomyces carlsbergensis*.

TABLE IV  
Response of Lactic Acid Bacteria to Added Potassium in Potassium-Deficient Medium\*

	K <sup>+</sup> per 10 cc			
	0 γ	100 γ	1000 γ	10,000 γ
	Galvanometer readings			
<i>Lactobacillus arabinosus</i>	100	94	35	33
“ <i>delbrueckii</i>	84	67	35	32
“ <i>casei</i>	81	62	28	25
<i>Streptococcus faecalis</i>	88	80	68	65
<i>Leuconostoc mesenteroides</i> P-60	94	73	69	69
“ “ 9135	96	88	59	53
<i>Lactobacillus pentosus</i>	93	67	35	35
“ <i>fermenti</i>	89	70	57	57

\* The basal medium, with potassium salts omitted, was pretreated once with *Lactobacillus arabinosus*.

The requirements of *Lactobacillus casei* and *L. delbrueckii* appear somewhat less than for *L. arabinosus*, and growth of these organisms occurred to a slight extent in the tubes without added potassium. This is undoubtedly due to residual traces of potassium in the medium, since on longer incubation organisms such as *L. arabinosus* also show some growth in the “blank” tubes (Table V). Without added potassium, however, growth does not reach a maximum, regardless of the period of incubation.

The amount of potassium required for maximum growth of these organisms agrees well with that found by others (4, 5) with different media. However, this amount is somewhat higher than one would expect to be required for direct metabolic use. Preliminary experiments indicate that factors other than the amount thus utilized contribute to the high requirement.

*Iron*—In no case did omission of iron from the medium affect growth deleteriously. Pretreatment of the medium with *Saccharomyces carlsbergensis* reduced the amount of iron to a level slightly below that required for optimal growth of this organism, but all of the lactic acid bacteria grew optimally on this medium without addition of iron. From the known facts that lactic acid bacteria grow anaerobically, do not contain cytochrome, and are catalase-negative, it would be expected that if iron is required at all, it would be required in extremely small amounts.

TABLE V

*Relation of Incubation Time to Potassium Requirement of Lactobacillus arabinosus*

K <sup>+</sup> per 10 cc	Galvanometer readings		
	24 hrs	47 hrs	95 hrs
7			
0	90	76	72
10	82	72	69
50	74	64	61
100	69	58	54
500	41	31	28

*Production of Mineral Deficiencies by Use of Citrate*—Although sodium or potassium citrate has been used as a buffer in media for *Streptococcus faecalis* (13), its addition to media for certain other lactic acid bacteria is known to inhibit growth (11). The ability of citrate to form non-ionic complexes with certain metallic ions has long been recognized (*e g* (14)), and a closer investigation of its inhibitory action on microorganisms seemed warranted.

Such investigation soon showed that inhibition could be prevented by addition of extra mineral salts to the medium, and that the effective ions were  $Mn^{++}$  and  $Mg^{++}$ . Illustrative data are given in Tables VI to IX. These data were obtained on the basal medium described earlier, but with both  $Mn^{++}$  and  $Mg^{++}$  omitted, and pretreated once with *Lactobacillus arabinosus* to remove traces of  $Mn^{++}$ . The growth response of *L. arabinosus* and *L. casei* to  $Mn^{++}$  in the presence and absence of added  $Mg^{++}$  is shown in Table VI. The response of *L. arabinosus* to  $Mn^{++}$  is essentially unchanged by addition of amounts of  $Mg^{++}$  up to 3.0 mg per 10 cc. As noted earlier

(Table III), growth of *L. casei* is enhanced by  $Mg^{++}$ , but the amount of  $Mn^{++}$  necessary to achieve maximum growth is nearly the same whether or not  $Mg^{++}$  is added (Table VI). In the presence of citrate, these relationships are altered. With *L. arabinosus* (Table VII), much larger amounts of  $Mn^{++}$  are required to permit growth when citrate is present, when the

TABLE VI

*Response of Lactobacillus arabinosus and Lactobacillus casei to Additions of Manganese and Magnesium in Medium Deficient in These Ions\**

		$Mn^{++}$ per 10 cc			
		0 $\gamma$	0.1 $\gamma$	1 $\gamma$	10 $\gamma$
		Galvanometer readings			
<i>L. arabinosus</i>	No added $Mg^{++}$	95	86	54	31
	3 mg $Mg^{++}$ added per tube	94	83	52	27
" <i>casei</i>	No added $Mg^{++}$	86	69	43	33
	3 mg $Mg^{++}$ added	80	61	34	26

\* The basal medium, with both manganese and magnesium salts omitted, was pretreated once with *Lactobacillus arabinosus*.

TABLE VII

*Effect of Varying Citrate and Magnesium Ion Concentration on Manganese Requirement of Lactobacillus arabinosus\**

		$Mn^{++}$ per 10 cc							
		0 $\gamma$	10 $\gamma$	50 $\gamma$	100 $\gamma$	150 $\gamma$	200 $\gamma$	250 $\gamma$	500 $\gamma$
		Galvanometer readings							
No citrate		99	29		24				
1% "		100		95	58	28	22	21	20
2% "		100			98	92	91	39	22
2% "	+ 100 $\gamma$ $Mg^{++}$	100			80	44	34	27	
2% "	+ 200 " "	100	75	43	36		24		
2% "	+ 300 " "	100	35	29			23		

\* Medium prepared as described in foot-note to Table VI. Citrate was added after pretreatment.

amount of citrate present is doubled, the amount of  $Mn^{++}$  necessary is doubled. With the addition of  $Mg^{++}$ , however, the amount of  $Mn^{++}$  required is greatly decreased, and with excess  $Mg^{++}$  present, the amount of  $Mn^{++}$  required for growth is only slightly larger when citrate is present than in its absence.

With *Lactobacillus casei* (Table VIII), a similar reciprocal relationship exists. In this case, however, manganese alone does not prevent inhibition



of growth by citrate, magnesium is also essential for growth. Magnesium is undoubtedly essential also in the absence of citrate, failure to observe more than a stimulatory effect probably reflects its incomplete removal from the medium.

A comparison of the relationship of other lactic acid bacteria to manganese, magnesium, and citrate is given in Table IX. All of the organisms grow well in the presence of citrate when both  $Mn^{++}$  and  $Mg^{++}$  are present, only *Streptococcus faecalis* grows in the absence of these added ions. The other organisms resemble either *Lactobacillus arabinosus* or *L. casei* in their behavior, or are intermediate between them.

TABLE VIII

*Effect of Concentration of Manganese and Magnesium Ions on Inhibition of Lactobacillus casei by Citrate\**

$Mg^{++}$ per 10 cc	$Mn^{++}$ per 10 cc						
	0 $\gamma$	1 $\gamma$	10 $\gamma$	100 $\gamma$	500 $\gamma$	1000 $\gamma$	10 000 $\gamma$
	Galvanometer readings						
0	100				98	98	98
500	95				35	27	
1000	84	85	54	24	22	23	
1500	81	39	35	26	23		
$Mn^{++}$ per 10 cc	$Mg^{++}$ per 10 cc						
	0 $\gamma$	300 $\gamma$	350 $\gamma$	400 $\gamma$	500 $\gamma$		
1000	100	82	49	29	24		
1500	98	65	43	26	24		

\* The medium was pretreated as described in the foot note to Table VI. 2 per cent sodium citrate was added after pretreatment.

It is logical to assume, by analogy with its effects on blood coagulation, that citrate enhances the requirements of these organisms for manganese and magnesium because it forms complexes with these ions which are unavailable for growth. Known complexes of citrate with metallic ions decrease in stability as the pH is lowered (14). This is consistent with the observation that progressively less manganese is required to counteract the inhibitory effect of citrate as the initial pH of the medium is lowered (Table X). The formation of similar complexes with citrate could also explain the observation that magnesium exerts a sparing effect on the requirement for manganese (and vice versa) in the presence of citrate, but not in its absence. Other bivalent metallic ions which form complexes with citrate also should have some effect in this direction. Most of these are toxic, and could not

be adequately tested  $\text{Ca}^{++}$ , however, is about one-thirtieth as effective as  $\text{Mg}^{++}$  in this respect for *L. arabinosus*. An interesting incidental observation made in connection with these studies was that  $\text{Zn}^{++}$  and  $\text{Ni}^{++}$  showed

TABLE IX

*Effect of Citrate on Response of Several Lactic Acid Bacteria to Manganese and Magnesium\**

Mn <sup>++</sup> Mg <sup>++</sup>	Micrograms ion added per 10 cc						
	0 0	0 1000	0 10 000	1000 0	10,000 0	1000 1000	1 000 10 000
	Galvanometer readings						
<i>Lactobacillus casei</i>	100	99	89	98	99	32	26
" <i>arabinosus</i>	100	100	100	25	27	25	50
" <i>delbrueckii</i>	100	100	96	82	55	37	28
<i>Streptococcus faecalis</i>	63	62		56		42	55
<i>Leuconostoc mesenteroides</i> P-60	100	100	97	100	63	59	57
" " 9135	99	100	99	50	45	50	47
<i>Lactobacillus fermenti</i>	100	99	100	93	69	75	61
" <i>pentosus</i>	100	99	99	23	20	20	22

\* 2 per cent sodium citrate added to a medium prepared as described in the footnote to Table VI

TABLE X

*Effect of pH on Availability of Manganese to Lactobacillus arabinosus in Presence of Citrate\**

pH	Mn <sup>++</sup> per 10 cc					
	0 γ	100 γ	200 γ	300 γ	400 γ	600 γ
	Galvanometer readings					
7 0	99			48	34	27
6 5	100		97	55	33	25
6 0	100		85	41	26	23
5 5	100	91	48	29	24	22
5 0	100	48	29	28	27	27

\* 2 per cent sodium citrate added to the medium prepared as described in Table VI

much less toxicity in the presence of citrate than in its absence, whereas  $\text{Cu}^{++}$  was slightly more toxic when citrate was present

*Manganese Requirement of Streptococcus faecalis*—Although *S. faecalis* was stimulated by addition of  $\text{Mn}^{++}$ , both on citrate-free and citrate-containing media, good growth without its addition occurred in both instances. To determine whether manganese was essential, the double strength basal

medium was pretreated with *Lactobacillus arabinosus* for 24 hours. After removal of the cells, sodium citrate (4 per cent of the double strength medium) was added, and the medium was sterilized and inoculated with a saline suspension of washed *S. faecalis* cells. After 24 hours, during which considerable growth occurred, the cells were centrifuged out and this doubly pretreated medium used to determine the response of *S. faecalis* to manganese. The results are given in Table XI. The growth-enhancing effect of manganese additions is clearly visible. When growth ceased at about 72 hours, the cultures with added manganese had grown much more than the unsupplemented tubes. It is tentatively concluded that *S. faecalis*, too, requires manganese for growth, but in far smaller quantities than do the

TABLE XI

*Response of Streptococcus faecalis to Manganese in Pretreated Medium Containing Sodium Citrate\**

Incubation period	Mn <sup>++</sup> per 10 cc						
	0 $\gamma$	0.01 $\gamma$	0.03 $\gamma$	0.1 $\gamma$	0.3 $\gamma$	3 $\gamma$	100 $\gamma$
	Galvanometer readings						
hrs							
12	88	88	84	83	82	81	77
72	82	77	70	70	66	60	57
116	83	77	70	70	65	63	56

\* The basal medium, with manganese salts omitted, was pretreated once with *Lactobacillus arabinosus*, 4 per cent of sodium citrate was then added to the double strength medium, and the pretreatment repeated once with *Streptococcus faecalis*.

*lactobacilli*. This conclusion must remain tentative, however, since growth on this final medium was slow, and did not reach maximum levels even with added manganese.

*Phosphate Requirements of Lactic Acid Bacteria*—To establish the amount of phosphate necessary for growth, the basal medium was modified as follows. Sodium citrate was added at a level of 100 mg per 10 cc of final medium, phosphates were omitted, and 5 mg of magnesium sulfate and 5 mg of manganese sulfate were added per 10 cc of medium. The enzymatic casein digest was replaced by a pancreatic fibrin digest<sup>1</sup> at a level of 75 mg per 10 cc. The medium was pretreated once with *Lactobacillus arabinosus*.

<sup>1</sup> 120 gm of fibrin were suspended in 2 liters of 0.8 per cent NaHCO<sub>3</sub>. 1 gm of pancreatin in 20 cc of water was added, and the mixture incubated under toluene at 37° for 4 days. The hydrolysate was steamed at 100° for 30 minutes, cooled, and filtered. Precipitates were removed as they appeared when the pH was dropped to 3.5 with HCl. The hydrolysate was adjusted to pH 6.5. It contained about 17 mg of solids per cc.

The response to added  $\text{KH}_2\text{PO}_4$  was then determined for eight lactic acid bacteria (Table XII). A phosphate deficiency sufficient completely or nearly completely to inhibit the growth of all organisms except *Streptococcus faecalis*, *L. casei*, and *L. delbrueckii* was obtained. In general, these three organisms also showed a greater response to small additions of phosphate than did the other organisms. For maximum growth, most organisms require about 3 mg of  $\text{PO}_4^{3-}$  (equivalent to 4.39 mg of  $\text{KH}_2\text{PO}_4$ ) per tube. Separate experiments showed that prolonging the incubation period from

TABLE XII

Response of Eight Lactic Acid Bacteria to Additions of Phosphate in Phosphate-Free Medium\*

	$\text{PO}_4^{3-}$ per 10 cc				
	0 $\gamma$	150 $\gamma$	600 $\gamma$	1500 $\gamma$	3000 $\gamma$
	Galvanometer readings				
<i>Lactobacillus arabinosus</i>	97	96	38	25	23
“ <i>delbrueckii</i>	87	79	43	34	28
<i>Streptococcus faecalis</i>	86	82	67	63	62
<i>Lactobacillus casei</i>	78	51	27	21	19
“ <i>fermenti</i>	90	79	55	53	52
“ <i>pentosus</i>	98	80	38	30	28
<i>Leuconostoc mesenteroides</i> , P-60	97	75	45	40	38
“ “ 9135	99	94	62	50	52

\* The basal medium, with fibrin digest substituted for the casein digest and with phosphate omitted, was pretreated once with *Lactobacillus arabinosus* (see the text)

24 hours to as long as 95 hours decreased the phosphate requirement only very slightly

## DISCUSSION

It is evident from the results cited above that in some cases a deficiency in essential inorganic ions can be obtained in a complex medium by preliminary growth in the medium of an organism requiring the ion for growth. The ability of an organism to reduce the concentration of an essential element to a level which will no longer support growth of that organism depends upon the concentration of the ion present as a contaminant in the medium and on the magnitude of the requirement of the organism for maximum growth. If the former concentration is high relative to the latter requirement, a single pretreatment of the medium, and perhaps several successive pretreatments, will not render the medium free of the ion. It is also evident that a medium sufficiently free of an element to demonstrate the requirement for it by one organism may contain sufficient of the mineral

to permit growth of other organisms. *Lactobacillus casei* and *Streptococcus faecalis*, for example, grow fairly well on a medium which contains insufficient manganese to support growth of *Lactobacillus arabinosus*, although both of the former organisms also appear to require manganese for growth.

For successful application of the pretreatment procedure used above for the preparation of deficient media, it is apparent that the inorganic ion (or other substance) to be removed must be the sole essential substance present in limiting amounts. Otherwise, a multiple deficiency may be encountered. Similarly, the organism used for the absorption must not produce, during growth, substances which will inhibit growth of organisms subsequently

TABLE XIII

Comparison of Amounts of Inorganic Salts Commonly Employed in Assay Media with Amounts Necessary for Maximum Growth

	Amounts commonly employed in assay media*	Amounts found necessary for maximum growth in present study	
		Acetate buffer	2 per cent citrate buffer
Mn <sup>++</sup> , $\gamma$	32.5	10-100	10-1000†
Mg <sup>++</sup> , "	197.4	0	0-1500†
Fe <sup>++</sup> , "	20.1	0	0
K <sup>+</sup> , mg	7.35	10	10
Na <sup>+</sup> , $\gamma$	39.3		
PO <sub>4</sub> <sup>==</sup> , mg	12.4	3	3

\* Speakman's salts in the concentrations customarily employed in assay media.

† The amount of Mn<sup>++</sup> required in the presence of citrate depends on the amount of Mg<sup>++</sup> present. For *Lactobacillus casei*, at least 500  $\gamma$  of Mg<sup>++</sup> should be present per 10 cc (see the text).

introduced into the medium. With the media and organisms used in this work, only one possible example of interference from one of these sources was encountered, *viz* *Streptococcus faecalis* failed to grow optimally (after supplementation with manganese) on a medium which had been pretreated first with *Lactobacillus arabinosus*, then with *Streptococcus faecalis* itself. The reason for this is not yet known. In no other case was a depressing effect on growth of an organism by preliminary pretreatment of the medium with the same or another organism encountered.

It is highly probable that the lactic acid bacteria require inorganic ions in addition to those shown to be essential by this investigation. An absolute requirement for magnesium, for example, could not be demonstrated by the pretreatment procedure alone, and it was only through the complex-forming action of citrate that requirement for this cation could be demonstrated. No precautions have been taken in the present investigation to eliminate

the inorganic ions furnished by glassware, etc., to the organisms. The requirements demonstrated above, are, therefore, minimal requirements of the organisms tested, and probably represent those inorganic ions which are required in the largest amounts for growth.

It is interesting to compare the amounts of the inorganic ions commonly added to assay media with the amounts found necessary to promote optimal growth of the organism with the highest requirement in the present study. This comparison is made in Table XIII. For maximum growth in 24 hours, concentrations of manganese ion and of potassium ion previously used have been marginal for some organisms, the remainder appear to be present in adequate amounts. The figures in Table XIII were determined after a 24 hour incubation period, frequently longer incubation periods are used, which result in a slightly decreased requirement for the inorganic ions.

#### SUMMARY

Under favorable circumstances, growth of an organism in a complex medium can be used to remove completely from the medium traces of a substance essential for growth of that organism.

By this procedure it has been shown that  $Mn^{++}$  is essential for *Leuconostoc mesenteroides*, all lactobacilli, and probably for *Streptococcus faecalis*. Potassium ion is also required in comparatively large amounts by all lactic acid bacteria investigated. The level of phosphate required by lactic acid bacteria for growth in an adequately buffered medium has also been established.

With this procedure it was possible to show that magnesium stimulated growth, but not that it was essential. The amount of magnesium required for growth of the lactic acid bacteria is certainly less than that required by yeast. Addition of ferrous iron to an iron-low medium neither enhanced nor inhibited growth.

Citrate inhibits growth of lactic acid bacteria when added to the medium in large amounts. This inhibition of growth can be prevented by increasing the amounts of manganese and of magnesium present in the medium, and appears due to the action of citrate in forming complexes with essential metallic ions. For some organisms, manganese alone prevents the "toxic" action of citrate, for others, both manganese and magnesium are essential. The amount of manganese required is, however, always decreased by addition of magnesium. It can also be decreased by addition of other bivalent metallic ions, such as calcium, which form complex ions with citrate.

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# THE INHIBITION OF PTEROYLGLUTAMIC ACID CONJUGASE AND ITS REVERSAL THE EFFECT OF NUCLEIC ACID- AND SULFHYDRYL-COMBINING REAGENTS

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(Received for publication, June 18, 1947)

We have called attention to the presence in natural materials of an inhibiting substance or substances affecting pteroylglutamic acid (PGA) conjugase from hog kidney and have demonstrated the inhibitor content of yeast extract (1) Sims and Totter have found (2) that a glutamic acid polypeptide of *p* aminobenzoic acid from yeast was inhibitory toward PGA conjugase derived from chicken pancreas and rat liver These *in vitro* experiments have been supplemented by the observation that crude yeast extract reduces the expected excretion of PGA by normal persons following its ingestion with inhibitor-free PGA hexaglutamyl conjugate (PHGA) (3)

A further study of the PGA conjugase inhibitor has been made because of its wide distribution in natural materials and its possible physiologic significance Following preliminary work with concentrates of the inhibitor from natural substances, nucleic acid, either of the ribose or desoxyribose type, was found to be a strong *in vitro* inhibitor of PGA conjugase This conjugase-inhibiting action of nucleic acids was destroyed by treatment with specific enzymes, namely ribonuclease or thymonucleo-depolymerase, or by the addition of some reducing agents Further, substances known to inhibit enzymes with active sulfhydryl groups have been shown to inhibit PGA conjugase *In vivo*, certain preparations of nucleic acid have reduced the expected excretion of PGA when PHGA was administered to normal subjects The purpose of this paper is to describe our experiments leading to these findings

## EXPERIMENTAL

*Methods for in Vitro Experiments*—The conjugase preparation used for most of these experiments was made by extracting hog kidney with 3 volumes of water, as described previously (1) The substrate was either a concentrate of PHGA from yeast or crystalline PHGA<sup>1</sup> Enzyme incubations to test for inhibitory activity were generally carried out as follows 56.5  $\gamma$  of PHGA, equivalent to 20  $\gamma$  of PGA, were dissolved in 2.5 cc. of 1 per cent sodium acetate buffer of pH 4.5 An estimated

<sup>1</sup> Kindly supplied by Dr. J. J. Pflüger of these laboratories

amount of the substance to be tested as inhibitor and 0.1 cc of the hog kidney extract were added. The volume was made up to 5 cc and incubated 18 hours at 37° in a water bath. The PGA freed from its conjugate during this incubation was determined by assay with *Streptococcus faecalis*. Controls for the various substances used as inhibitors were set up without conjugase to detect any possible effect on *Streptococcus faecalis*.

*Concentration of Inhibitor*—The inhibition of PGA conjugase from hog kidney was found to be proportional to the amounts of natural inhibitors present in the incubation mixture, such as yeast extract or molasses. This is shown in Table I. Since molasses was low in PGA content, it was used as a source of the inhibitor for concentration. One lot of molasses was set aside to be used as a standard to compare the inhibitor content of different materials and to follow the concentration.

600 cc of molasses were dialyzed 72 hours in running tap water and then

TABLE I

*Relation of Degree of Inhibition of Pteroylglutamic Acid Conjugase to Amount of Natural Inhibitors Present*

Molasses	PGA released in 18 hrs	Yeast extract	PGA released in 18 hrs
cc	γ	mg	γ
0	15.5	0	14.0
0.02	11.5	5	6.0
0.04	7.5	10	2.5
0.06	6.0	15	1.5
0.1	4.5		
0.2	1.0		

concentrated *in vacuo*. This reduced the inhibitor content only slightly. 30.2 gm of  $(\text{NH}_4)_2\text{SO}_4$  per 100 cc were added to make 40 per cent saturation. After standing 2 hours the mixture was centrifuged at high speed and the liquid discarded. The precipitate was dissolved in water and again dialyzed against running water. This resulted in a 60-fold concentration. The concentrate gave positive Molisch and Bial reactions and the desoxy-ribonucleic acid content as determined by the diphenylamine reaction (4) was high. These findings led to the testing of nucleic acid as an inhibitor of PGA conjugase from hog kidney.

*In Vitro Experiments with Nucleic Acid As Conjugase Inhibitor*—Different samples of thymus nucleic acid and yeast nucleic acid were tested and found to be strong inhibitors of PGA conjugase from hog kidney. Thymus nucleic acid prepared by Hammarsten's method was the most powerful inhibitor studied. These data are shown in Table II. The assay of inhibitor content was made by incubating, simultaneously, three levels of

the material to be tested and four levels of the standard molasses with conjugate and PHGA substrate in the manner described above. Following this the amount of PGA released in each tube was assayed in the usual manner, the data for the standard were then plotted, and the concentration of inhibitor in the unknown determined from this curve.

The sodium salt of thymus nucleic acid was prepared according to the method of Hammarsten (5). This preparation gave a negative biuret test. No sulfur could be detected with a micromethod accurate to 0.003 per cent. Fig. 1 shows a good proportionality between the amount of nucleic acid used as inhibitor and the inhibition produced. This inhibition continues regardless of the length of incubation. For this experiment crystalline PHGA was used as the substrate, and the incubations were carried out as described.

Fig. 2 shows the reciprocal of the velocity of the reaction plotted against

TABLE II  
*Comparison of Inhibitor Activity of Some Natural Substances*

Source of inhibitor	Relative inhibitor activity*
Hammarsten's thymus nucleic acid	160
Levene's thymus nucleic acid	100
Yeast nucleic acid	79
Concentrate of inhibitor from molasses	60
Concentrate from Type III yeast extract	32
Liver extract (70% alcohol insoluble)	10
Type III yeast extract	7.6
Difco yeast extract	2

\* Molasses = 1

the reciprocal of the substrate concentration in the presence and absence of nucleic acid. Crystalline PHGA was also used for the substrate in this experiment. Each tube contained 0.02 cc. of the hog kidney extract, the substrate, and 2.5 cc. of 1 per cent acetate buffer of pH 4.5 in a total volume of 5 cc. The initial velocity was found by assaying samples taken at 30 minutes, 1 hour, and 2 hours. In this plot straight lines are formed, which is in accordance with Lineweaver and Burk's proof (6) of competitive inhibition. From this we can conclude that an enzyme-inhibitor complex is formed which is not reversed during the reaction.

The relation of viscosity to inhibition was studied. Solutions of gelatin and of agar which were much more viscous than the nucleic acid solution did not inhibit PGA conjugase. Table III shows that the viscosity of nucleic acid can be changed by non-enzyme methods without significantly altering its conjugase-inhibiting action.

The effect of nucleic acid on PGA conjugase from several tissues other than hog kidney was studied and the results are shown in Table IV. The reactions were carried out at the optimum pH for each enzyme. In the case of rat liver conjugase pH 7.6 was used in addition to the optimum pH of 4.5. Comparatively little enzyme activity for PHGA is evidenced at this pH, which is contrary to reports in the literature. However, in these reports inhibitor-containing yeast extracts were used as substrates. It is

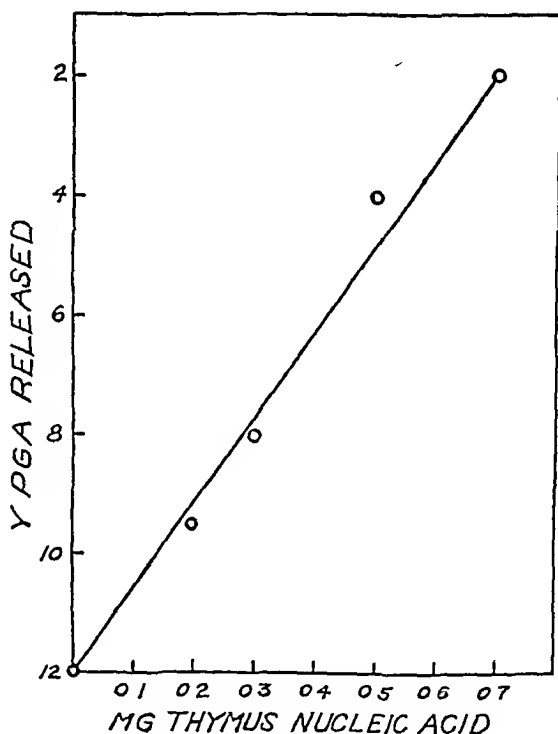


FIG. 1. Inhibiting effect of Hammarsten's thymus nucleic acid on hog kidney conjugase. The reaction mixtures contained 56.5  $\gamma$  of PHGA, 2.5 cc. of 1 per cent sodium acetate buffer of pH 4.5, 0.1 cc. of conjugase solution, and the indicated amounts of thymus nucleic acid.

likely that at pH 7.6 the depolymerase enzyme (optimum pH 7.1) contained in the crude rat liver preparations destroyed the inhibitor, with a resultant greater release of PGA than when the reaction was carried out at pH 4.5, at which point the depolymerase activity would be negligible.

The chicken pancreas enzyme was purified by the method of Mirs and Laskowski (7). This purified enzyme has an optimum pH of 7.8 and very little activity at pH 4.5.

*Inhibitor Action of Enzymes*—The inhibiting action of thymus nucleic acid was destroyed by treatment with thymonucleodepolymerase

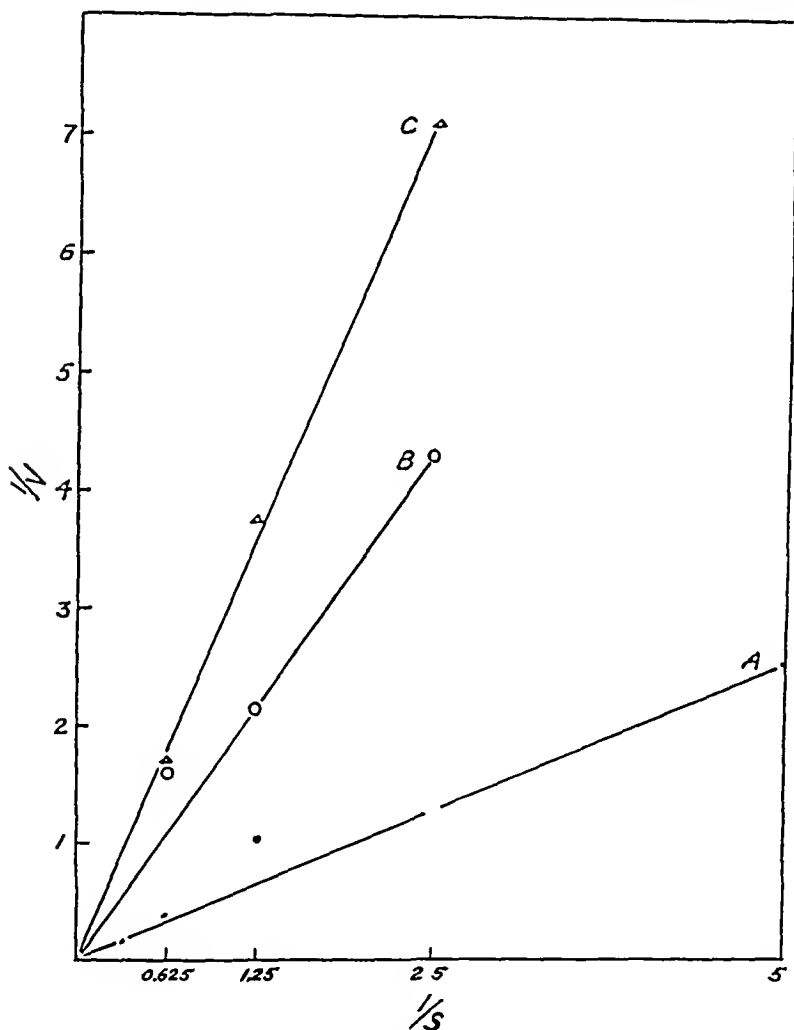


FIG 2 Velocity of PGA conjugase from hog kidney acting in mixtures of substrate and thymus nucleic acid in different proportions. Curves A, B, and C represent reaction mixtures containing 0.01, 0.05, and 0.1 mg of thymus nucleic acid respectively.  $1/S$  represents the reciprocal of the substrate concentration (molar concentration of PHGA) given in millions,  $1/V$  represents the reciprocal of the velocity (moles of PGA released per hour per mole of PHGA) given in tens of millions.

and that of yeast nucleic acid by ribonuclease. Also the inhibitory content of crude yeast extract was greatly lowered by treatment with these enzymes.

TABLE VII

*Inhibition of Conjugase by Sulphydryl-Combining Reagents and Reversal with Cysteine*

Inhibitor present	Concentration of inhibitor	PGA released in 18 hrs			
		Experiment 1		Experiment 2	
		No reducing agent added	Cysteine added*	No reducing agent added	BAL added†
	M	per cent of maximum	per cent of maximum	per cent of maximum	per cent of maximum
None		100		100	
3-Amino-4-hydroxybenzenear-senous acid	0.001	8	50	0	15
	0.0001	60	100		
Arsenic trioxide	0.001	50	100	20	84
p-Arsenosobenzoic acid	0.001	60	100	12	100
4-Arsenosophenylurea	0.001	60	100	12	100
Disodium 2-carboxy-4-hydroxy-mercuriphenoxacetate	0.0001	0	50	19	80
Sodium bromoacetate	0.001	8	50	21	96

\* Cysteine was added in an amount equal to 10 times the molar concentration of the inhibitor used

† BAL (2,3 dimercaptopropanol) was added in an amount equal to 5 times the molar concentration of the inhibitor used

TABLE VIII

*Effect of Nucleic Acid on Urinary Excretion of Pteroylglutamic Acid Following Ingestion of Pteroylhexaglutamylglutamic Acid*

Treatment	Excretion per 24 hrs		
	Subject 1	Subject 2	Subject 3
2 mg PHGA*	γ	γ	γ
2 " " + 1 gm thymus nucleic acid (Hammarsten)*	593	426, 495	439, 339
	662, 522	573, 290	391
2 mg PHGA + 1 gm thymus nucleic acid (Levene)	78	150	123
2 mg PHGA + 1 gm depolymerized thymus nucleic acid (Levene)	843		
4 mg PHGA			1650
4 " " + 2 gm yeast nucleic acid (Schwarz)			1592

\* When two excretion values occur in a column they represent repeated treatment on the same individual at various time intervals

demonstrate the sulphydryl groups in an enzyme were found to inhibit PGA hog kidney conjugase. These results are shown in Table VII. The inhibition could be reversed by cysteine and 2,3-dimercaptopropanol.

(BAL) For this experiment the compounds were added directly to the enzyme extract and allowed to stand 10 minutes, then aliquots were transferred to the tubes containing cysteine or BAL, the substrate, and buffer mixture

*In Vivo Experiments with Nucleic Acid As Conjugase Inhibitor*—Previous work (3) has suggested that the possible effect of PGA conjugase inhibitors *in vivo* can be studied by determining whether these substances when administered with PHGA lower the expected urinary excretion of PGA. Since nucleic acid has now been found to be an inhibitor of PGA conjugase *in vitro*, its *in vivo* action was tested by this method

The data from Table VIII show that when thymus nucleic acid prepared by the Levene method and PHGA were administered to normal subjects the urinary excretion levels were lower than when PHGA was given alone. Thymus nucleic acid purified according to Hammarsten and a commercial sample of yeast nucleic acid did not consistently have this effect on PGA excretion levels. When thymus nucleic acid (Levene) was treated with thymonucleodepolymerase, its apparent action *in vivo* on PGA conjugase systems was destroyed, as evidenced by the fact that it no longer affected PGA excretion following PHGA administration

#### DISCUSSION

Greenstein and Chalkley (13) and also Zittle (14) have found that nucleic acid inhibits certain reducing enzymes. The inhibition of PGA conjugase from various tissue sources by nucleic acid as reported here indicates that its inhibitory effect is not confined to the enzymes concerned with reduction, since PGA conjugase is considered to be a hydrolytic enzyme (15). Unlike the inhibitions described by Greenstein and Chalkley and Zittle, the inhibiting action of nucleic acid toward PGA conjugase is removed by specific depolymerizing enzymes. Thymonucleodepolymerase removed the inhibiting activity of thymus nucleic acid but had no effect on yeast nucleic acid, while crystalline ribonuclease inactivated yeast nucleic acid and not thymus nucleic acid. These observations indicate that the nucleic acid molecule itself and not a contaminant is responsible for PGA conjugase inhibition.

The reduction in inhibiting action of yeast extract by depolymerases is evidence that nucleic acid is the substance in yeast extract that is in large part responsible for the *in vitro* inhibition of PGA hog kidney conjugase by yeast extract. It is not readily apparent whether the PGA conjugase inhibitor studied by Sims and Totter (2), in the concentration present in yeast extract, affects hog kidney conjugase.

Greenstein and Chalkley (13) noted a decrease in nucleic acid inhibition for reducing enzymes after treating highly polymerized nucleic acid with

hydrosulfite We find that both thymus nucleic acid and yeast extract lose their inhibitory action against PGA conjugase after being treated with reducing agents

The inhibition of PGA conjugase with sulfhydryl-combining reagents and the reversal of this inhibition with cysteine and BAL indicates that PGA conjugase from hog kidney probably contains an active sulfhydryl group It might be concluded that the enzyme-inhibitor complex which forms with nucleic acid involves this group The presence of a sulfhydryl group could explain why there was a large loss of activity on attempting to purify this enzyme (1)

It has not been possible to demonstrate the inhibition of purified PGA conjugase from chicken pancreas by nucleic acid The suggestion that this conjugase from chicken pancreas is an entirely different enzyme than that found in the other tissues has already been made (1, 7, 16) The fact that conjugase from chicken pancreas is not inhibited by nucleic acid adds evidence to this view

It has been shown that Levene's thymus nucleic acid reduces PGA excretion from expected levels following its ingestion with PHGA and that this effect is destroyed by treatment with depolymerase enzyme This is some evidence that a nucleic acid in its polymerized form has an *in vivo* effect on conjugate cleavage However, other preparations of thymus nucleic acid and also yeast nucleic acid, although they are particularly active *in vitro* against PGA conjugase, have apparently no effect *in vivo* No explanation can be given for the varying action of these different nucleic acids on PGA conjugase systems *in vitro* and *in vivo*

We have not been able to destroy completely the *in vivo* PGA conjugase inhibition of yeast extracts by treatment with the depolymerases, and it is possible that other inhibitors with *in vivo* action are present

These studies, showing the *in vitro* and the less well defined *in vivo* effect of nucleic acid on PHGA cleavage, present the possibility that, since nucleic acid is a universal cellular constituent and since PGA occurs in body tissue mostly in the conjugated form, nucleic acid of some type may be a regulating mechanism for the release of free PGA within the cell

We have found that the results of the experiments reported here can be used to advantage in the microbiological assay of PGA in natural materials, which contain the vitamin largely in a conjugated form The most effective method of releasing PGA from its conjugate is by enzymatic treatment with a source of conjugase, a procedure that is greatly influenced by the presence of conjugase inhibitors (1) These studies indicate that the effect of nucleic acid as a conjugase inhibitor can be negated either by treatment with specific enzymes or, more practically, by the addition of cysteine or hydrogen sulfide



## SUMMARY

The activities of pteroylglutamic acid conjugase inhibitor of several natural substances were compared *in vitro*. A concentrate of the inhibitor prepared from molasses was found to contain a large proportion of nucleic acid. Thymus and yeast nucleic acids were shown to be strong inhibitors of PGA conjugase from hog kidney, hog intestine, rat liver, and human leucocytes. The inhibition is competitive in nature.

Nucleic acid did not inhibit purified PGA conjugase from chicken pinnreas.

The inhibiting effects of thymus nucleic acid, yeast nucleic acid, and yeast extract were destroyed by incubation with depolymerizing enzymes or by treatment with reducing agents.

The presence of an active sulfhydryl group in PGA conjugase from hog kidney was indicated.

*In vivo* PGA conjugase inhibition by nucleic acids did not exactly parallel the inhibition observed *in vitro*. However, the nucleic acid preparation which inhibited *in vivo* PGA conjugase, as evidenced by decreased PGA excretion levels, showed no inhibiting effect after treatment with depolymerizing enzyme.

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## COBALT METABOLISM STUDIES

### IV TISSUE DISTRIBUTION OF RADIOACTIVE COBALT ADMINISTERED TO RABBITS, SWINE, AND YOUNG CALVES\*

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(Received for publication, May 17, 1947)

Investigations on the role and behavior of cobalt in the animal body have been undertaken in this laboratory, owing primarily to recognition of cobalt as a trace dietary essential in the ruminant diet. The employment of radioactive isotopes in this study has overcome many of the inherent limitations imposed by the use of chemical methods with a system wherein such a small amount of the element is physiologically involved.

The metabolic role of cobalt presents many interesting aspects. A species difference is suggested by the observation that, while cobalt is a dietary essential for the ruminant, there is no definite evidence that such is the case for the non-ruminant (1). Although it is not known whether cobalt is essential for the normal functioning of the hematopoietic organs, the well known action of cobalt in stimulating erythrocyte formation in non-ruminants (2) indicates that a mechanism does exist whereby cobalt could be effective in this manner. Recently the toxicity of cobalt to workers in the steel industry has become an important problem requiring knowledge as to cobalt distribution in the body. In general, data on the tissue distribution of radioactive materials in animals must always serve as a starting point for consideration of possible therapeutic and diagnostic uses, and the present information on cobalt may be of value in that connection.

Previous publications in this series (3-5) have described procedures with radioactive cobalt for large as well as small animals and have presented data on the excretion and tissue distribution of cobalt administered to mature cattle. When the labeled cobalt was injected into the jugular vein, the accumulation in all of the tissues was sufficient for satisfactory measurement. However, practically none of the orally administered cobalt was detected in the various tissues, owing to poor absorption from the tract and the fact that the amounts of radioactive cobalt used were small in relation to the size of the animals, which weighed up to 600 pounds.

This paper reports findings with young calves in which larger activities per unit of body weight were employed so that a more complete picture was

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obtained as to the tissue distribution of cobalt absorbed from the tract. Similar data on swine and rabbits are presented for species comparison.

### EXPERIMENTAL

The radioactive cobalt was supplied by the Radioactivity Center of the Massachusetts Institute of Technology through the courtesy of Dr. Robley D. Evans and Dr. John W. Irvine, Jr. It was prepared by bombardment of iron with deuterons, and was obtained as a purified solution of cobalt chloride, consisting of a mixture of three cobalt isotopes having a half life ranging from 65 to 270 days. The preparation contained 3 mg. of cobalt with an initial specific activity of 10,680 micrograms ( $\gamma$ ) of radium  $\gamma$ -ray equivalent per mg. The measurements here reported were sensitive to 0.0002 to 0.0004  $\gamma$  of cobalt. The samples were ashed, the ash brought into solution, and the activity measured with a dipping type Geiger-Müller counter tube, the details of this procedure have been described earlier (5).

### Results

*Tissue Distribution in Young Calves*—Two normal 30-day-old Jersey bull calves, each weighing 47 pounds, were used in this study, they were on a ration of whole milk. One was given a jugular injection of 60  $\gamma$  of radioactive cobalt in 1 ml. of solution and sacrificed after 17 hours, while the other received the same dose orally in the milk and was sacrificed after 5 days. The procedure for the slaughter of the animals and the handling of the tissues has been described in an earlier publication (3).

Table I presents the tissue concentrations obtained and the percentages of the dose contained in the whole tissue or organ. The concentration values have been calculated on the basis of 100  $\gamma$  of cobalt dosage for comparative purposes. The distribution of the injected cobalt is in most respects similar to that reported previously for the mature animals (5). The cobalt was almost completely removed from the blood, the pathway being indicated by the high concentrations found in the urine, kidney, liver, and bile. The percentages of the dose in the blood and liver were 0.43 and 25.6 respectively, which are of the same order of magnitude as previously reported for mature animals. The highest concentrations were found in the liver, adrenals, thyroid, intestinal lymph glands, lung, pancreas, and spleen. Only small amounts were found in the brain, eye, muscle, bone, and teeth.

The cobalt concentration in the pancreas was about one-fourth that in the liver and about twice that in the spleen. The concentration in the red bone marrow was about one-half that in the spleen and 3 times that of the white bone marrow. The ligament and cartilage showed greater cobalt accumulation than did the muscle tissue. The abomasum showed the highest con-

TABLE I

*Distribution of Labeled Cobalt Administered to Young Calves (Dosage 60  $\gamma$  of Cobalt)*

Tissue	Injection (sacrificed after 17 hrs.)		Oral administration (sacrificed after 5 days)	
	$\gamma$ per 100 gm fresh weight per 100 $\gamma$ dosage	Per cent of dose in whole tissue	$\gamma$ per 100 gm fresh weight per 100 $\gamma$ dosage	Per cent of dose in whole tissue
Pituitary	*	0	*	0
Thyroid	0.47	0.017	0.12	0.0047
Thymus	0.22	0.093	0.038	0.021
Adrenals	0.80	0.030	0.24	0.0083
Reproductive organs	0.18	0.044	0.044	0.011
Brain	0.038	0.093	0.014	0.031
Eye	0.049	0.023	0.012	0.0039
Intestinal lymph glands	0.33		0.075	
Heart	0.21	0.30	0.065	0.095
Blood	0.035	0.43	0.012	0.19
Aorta	0.20	0.034	0.070	0.039
Lung	0.42	1.65	0.15	0.79
Trachea	0.24	0.34	0.11	0.076
Kidney	0.97	1.85	0.17	0.33
Bladder	0.19	0.065	0.025	0.0048
"    urine	38.3	46.7		
Tongue	0.089	0.057	0.015	0.016
Esophagus	0.11	0.038	0.032	0.087
Abomasum	0.33	0.54	0.060	0.087
"    contents	0.0089	0.030	0.0011	0.006
Reticulum	0.16	0.052	0.066	0.023
Omasum	0.23	0.098	0.061	0.021
Rumen	0.12	0.17	0.040	0.045
"    and reticulum contents	0.0049	0.032	0.028	0.12
Small intestine	0.23	1.62	0.043	0.36
"    "    contents	0.20	1.15	0.033	0.097
Large "    "	0.21	0.78	0.030	
"    "    contents	0.86	0.88	0.082	0.12
Pancreas	0.81	0.25	0.085	0.018
Spleen	0.44	0.20	0.070	0.054
Liver	3.56	25.6	0.45	2.48
Gallbladder and Bile	4.16	0.14	0.063	0.0027
Tenderloin, muscle	0.040		0.0078	
Gastrocnemius muscle	0.036		0.0078	
Ligament (nuchal)	0.11		0.018	
Cartilage (costal)	0.16		0.11	
Bone (femur)	0.039		0.022	
Red bone marrow (ribs)	0.25		0.054	
White bone marrow (long bones)	0.076		0.023	
Teeth	0.17		0.12	

\* Amount in sample less than 0.0004  $\gamma$

centration for tissues of the tract, the concentrations in the omasum and small and large intestines being slightly lower, the lowest values being found in the rumen and reticulum. Very little cobalt was found in the stomach contents.

The calf receiving the cobalt orally was not killed until 5 days after administration in order to permit maximum absorption from the tract. The liver accumulated 2.48 per cent of the dose, which was considerably higher than the maximum value of 0.42 per cent found with mature animals. While the absorption may have been influenced by the fact that the cobalt was fed in milk, studies with rats have shown no increased absorption when the cobalt was administered in milk compared with water. The concentrations and percentages of the dose in the whole tissue were one-half to one-tenth the corresponding injection values. The proportional distribution of the absorbed ingested cobalt was similar to that of the injected cobalt. This ingestion experiment was repeated on another month-old calf with results essentially as reported in Table I, which are omitted for brevity.

In the case of the calves described above and many of the cattle used in earlier studies, representative samples were taken from the following sections of the tract: the fundus and pyloric regions of the abomasum, the duodenum, jejunum, and ileum. When the cobalt was orally administered, the concentration in the pyloric section was slightly higher than in the fundus section. However, in the injection studies, there was no consistent trend. With both ingested and injected cobalt the concentrations in the three regions of the small intestine were very nearly the same, however, the jejunum consistently showed lower values than the duodenum or ileum.

With each of the tissues mentioned above and also the large intestine, the inner surface was removed mechanically and designated as the mucosa, the remainder being designated as muscular tissue. When the cobalt was orally administered, the concentrations in the mucosa averaged 1.5 times those in the corresponding muscular tissues, while with the injected cobalt, the average ratio was 2.1. It is difficult to ascertain the extent to which the abomasum and intestines may secrete injected cobalt into the lumen owing to the relatively large amounts carried by the bile.

*Tissue Distribution in Swine*—Two normal pure bred Duroc gilts, about 6 months old, weighing 100 to 120 pounds, were used in this investigation. One received 30  $\gamma$  of radioactive cobalt in 0.5 ml. of solution by injection into a vein of the hind leg and was sacrificed after 23½ hours, while the other was given 60  $\gamma$  by capsule and was sacrificed after 4 days. The animals were slaughtered and the tissues handled in essentially the same way described previously.

Table II presents values for the concentrations in the tissues of swine

TABLE II  
Distribution of Labeled Cobalt Administered to Swine

Tissue	Injection*		Oral administration†	
	γ per 100 gm fresh weight per 100 γ dosage	Per cent of dose in whole tissue	γ per 100 gm fresh weight per 100 γ dosage	Per cent of dose in whole tissue
Pituitary	‡	0	‡	0
Thyroid	0 11	0 0044	‡	0
Thymus	0 038	0 011	0 0086	0 0043
Adrenals	0 38	0 012	0 020	0 00067
Reproductive organs	0 087	0 096	0 0041	0 0087
Brain	0 012	0 015	0 0022	0 0022
Eye	0 023	0 0033	0 0051	0 00072
Intestinal lymph glands	0 10		0 0062	
Heart	0 081	0 14	0 0069	0 017
Blood	0 068	1 48	0 0020	0 065
Aorta	0 058	0 018	0 0070	0 0022
Lung	0 078	0 45	0 0058	0 037
Trachea	0 052	0 012	0 0059	0 0054
Kidney	0 60	0 76	0 035	0 072
" fat	0 011		0 0020	
Bladder and urine	0 060	0 017	0 0032	0 0014
Tongue	0 034	0 049	0 0021	0 0034
Esophagus	0 12	0 061	0 011	0 0064
Cardiac stomach, mucosa	0 11		0 0070	
" " muscular	0 052		0 0050	
Fundus " mucosa	0 14	0 45	0 0038	0 029
" " muscular	0 035		0 0040	
Pyloric " mucosa	0 075		0 0049	
" " muscular	0 041		0 0053	
Stomach contents	0 0030	0 085	0 00060	0 0095
Duodenum, mucosa	0 090		0 0073	
" muscular	0 084		0 0063	
Jejunum, mucosa	0 077	1 7	0 0043	0 070
" muscular	0 067		0 0051	
Ileum, mucosa	0 11		0 0084	
" muscular	0 080		0 0057	
Small intestine contents	0 024	0 35	0 0043	0 068
Large " mucosa	0 12	0 64	0 014	0 099
" " muscular	0 049		0 0029	
" " contents	0 12	1 3	0 025	0 68
Pancreas	0 22	0 20	0 0079	0 019
Spleen	0 13	0 068	0 0064	0 0067
Liver	0 30	3 7	0 043	0 60
Gallbladder	0 098	0 0052	‡	0
Bile	0 071	0 012	0 017	0 0039

TABLE II—*Concluded*

Tissue	Injection*		Oral administration†	
	$\gamma$ per 100 gm. fresh weight per 100 $\gamma$ dosage	Per cent of dose in whole tissue	$\gamma$ per 100 gm. fresh weight per 100 $\gamma$ dosage	Per cent of dose in whole tissue
enderloin, muscle	0 021		0 0028	
Gastrocnemius muscle	0 022		0 0015	
Cartilage	0 035		0 0043	
Bone	0 018		0 00083	
Red bone marrow (ribs)	0 063		0 0062	
White bone marrow (long bones)	0 039		0 0032	
Teeth	†	0	0 04	

\* Received 30  $\gamma$  of cobalt, sacrificed after 23½ hours† Received 60  $\gamma$  of cobalt, sacrificed after 4 days‡ Amount in sample less than 0 0002  $\gamma$ 

TABLE III

*Distribution of Injected Labeled Cobalt in Rabbits, in Per Cent of Administered Dose (2 4  $\gamma$  of Cobalt)*

Tissue	Time following injection				
	1 hr	5 hrs	16 hrs	48 hrs	159 hrs
Thyroid	0*	0	0	0	0
Thymus	0 13	0 05	0 05	0 05	0 05
Adrenals	0 02	0	0	0 01	0
Reproductive organs	0 52	1 0	0 16	0 11	0 11
Eyes	0 07	0 04	0 06	0 03	0
Heart	0 39	0 41	0 28	0 19	0 03
Blood	2 4	3 0	2 3	1 3	0 19
Lung	0 8	0 7	0 4	0 3	0 2
Kidney	3 2	2 4	0 9	1 1	0 4
Bladder and urine	13 0	44 1	0 18	0 5	0
Stomach (cardiac)	1 0	0 7	0 6	0 4	0 2
“ (pyloric)	0 5	0 3	0 2	0 2	0 1
“ contents	0 08	0 07	1 6	0 6	0 7
Small intestine and contents	1 6	2 0	1 5	0 9	0 49
Large “ “ “	2 1	4 8	8 0	10 0	3 8
Pancreas	0 3	0 2	0 1	0 09	0 02
Spleen	0 1	0 02	0 02	0 05	0 04
Liver	16 5	12 7	8 9	5 0	3 4
Gallbladder and bile	0 05	0 08	0 07	0 18	0

\* Amount in sample less than 0 0003  $\gamma$ 

and the percentages of the dose contained in the whole tissue or organ  
cobalt distribution was quite similar to that observed with cattle

The  
The



amount of injected cobalt found in the liver was less than the corresponding values for both young and mature cattle, and the percentage of ingested cobalt found in the liver was about the same as with the older cattle, but lower than the value for the young calf. The relative concentrations of injected cobalt in the pancreas, spleen, and red bone marrow were essentially the same as with the calf, although the liver value was proportionately low. Here again the injection values were 2- to 10-fold higher than the corresponding ingestion values.

TABLE IV  
*Distribution of Orally Administered Labeled Cobalt in Rabbits, in Per Cent of Administered Dose (60  $\gamma$  of Cobalt)*

Tissue	Time following oral administration				
	5 hrs	24 hrs	96 hrs	144 hrs	264 hrs
Thyroid	0*	0	0	0	0
Thymus	0	0 013	0	0	0
Adrenals	0	0 0017	0 12	0	0
Reproductive organs	0	0 013	0 022	0	0
Heart	0	0 036	0 0072	0	0
Blood	0 0017	0 23	0 033	0	0
Lung	0 0053	0 058	0 38	0	0
Kidney	0 0033	0 16	0 098	0 015	0 0061
Bladder	0 0014	0 046		0	0
"    urine	0 073	0 065			0
Stomach (cardiac)	0 0026	0 042	0 032	0 0039	0 0022
"    (pyloric)	0 0094	0 019	0 017	0	0
"    contents	0 019	0 0050	0 11	0 78	0 46
Small intestine	0 043	0 15	0 033	0 70	0 014
"    "    contents	0 023	0 0036	0 0066	0 12	0 027
Large "    "	0 043	0 077	0 0075	0 24	0 040
"    "    contents	26 3	0 102	0 50	2 58	1 50
Spleen	0	0 0053	0 041	0	0
Liver	0 019	0 47	0 42	0 050	0 026
Gallbladder and bile	0	0 0021	0	0	0

\* Amount in sample less than 0 0003  $\gamma$

There seemed to be only slight differences in the cardiac, fundus, and pyloric sections of the stomach, however, as with the cattle, the jejunum showed lower values than the duodenum and the ileum. In general, the ratio of mucosal to muscular concentration was higher for injected cobalt than for ingested cobalt, which agrees with the data from cattle.

*Tissue Distribution in Rabbits*—Eighteen pedigreed Dutch breed rabbits on a normal ration, about 7 months old, weighing about 1500 gm, were used in this work. Tables III and IV show the percentages of injected and in-

gested cobalt contained in the whole tissue or organ at varying times after administration of the dose. In many cases the values are averages from two animals. For injection into the ear vein, a dosage of 2.4  $\gamma$  of cobalt in 0.5 ml. of solution was used, while for oral administration 60  $\gamma$  of cobalt in 1 ml. of solution were given by stomach tube. These animals were fasted for about 18 hours before administration of the cobalt.

Here again there is the same pattern that was observed with cattle and swine. After 1 hour there was 2.4 per cent of the injected cobalt in the blood, the amount decreasing to 0.19 per cent after 159 hours, the corresponding values for the liver were 16.5 and 3.4 per cent. Very little injected cobalt reached the stomach contents and its appearance in the intestines and contents coincided with the disappearance from the liver. In agreement with earlier findings the pancreas showed greater initial accumulation but more rapid loss of cobalt, presumably by secretion into the tract, than did the spleen. The values in Table IV indicate that the ingested cobalt was poorly absorbed and almost completely eliminated within the first 24 hours. The maximum accumulation in the liver of 0.47 per cent of the ingested dose agrees with the values found for mature cattle and swine.

#### DISCUSSION

The data here presented, supplementing earlier reports (3-5), lead to the following picture of cobalt partition by cattle. Approximately 95 per cent of the cobalt injected into the jugular vein is removed from the blood within a matter of minutes, that which remains is found almost entirely in the plasma. The liver may retain up to 46 per cent of the dose, a large proportion of which is secreted in the bile. About 65 per cent of the dose is ultimately eliminated in the urine and about 30 per cent in the feces. In mature animals only traces of injected cobalt, probably carried by the saliva, reach the rumen, reticulum, or omasum contents, while small amounts are found in the abomasum contents. The abomasum usually shows the highest concentration of the stomach tissues. The mucosa of the abomasum and intestines show a selective accumulation, which could be due to contact with the cobalt in the bile, or which might be taken to indicate that these tissues secrete injected cobalt into the lumen. If the former view is the correct one, it would be necessary to postulate that considerable amounts of bile reach the abomasum contents, and that absorption of biliary cobalt differs from that of ingested cobalt, since mucous accumulation of ingested cobalt is lower than that of injected cobalt, and the amount of ingested cobalt passing through the tract is at least 2.5 times that of biliary cobalt.

Injected cobalt is retained by practically all the tissues, the higher concentrations being found in the glandular organs, particularly the adrenals,

thyroid, liver, thymus, intestinal lymph glands, and pancreas. The maximum amount found in the pancreas was 0.6 per cent of the dose, so that, while the initial tissue concentration may be relatively high, the actual amount reaching the tract by this path will be small compared with the biliary cobalt secretion, these findings agree with those of Shelne, Chaikoff, and Montgomery (6), who reported that 0.3 per cent of the radiocobalt injected into a dog appeared in the external secretion of the pancreas. The concentration in the spleen is usually lower than that of the pancreas and somewhat higher than that of the red bone marrow. The consistently high values in the lymph glands indicate the importance of the lymph system in the transport of cobalt.

Cobalt accumulation is low in such tissues as the muscle, bone, ligament, cartilage, eye, and white bone marrow. Unpublished results<sup>1</sup> with young calves sacrificed 5 minutes, 1 hour, 6 hours, and 12 hours after injection, show that considerable accumulation may occur initially in the brain, with subsequent rapid disappearance, which would account for the low values reported in this series of papers, negative results for the pituitary are due in part to the small sample available.

Orally administered cobalt is poorly absorbed, as is indicated by the low tissue concentrations found and the small amounts appearing in the urine, mature cattle eliminated less than 1 per cent of the dose by this path. The liver accumulates the major portion of the absorbed cobalt and is apparently effective in minimizing general distribution, with older animals the maximum percentage of the cobalt dose found in the liver was 0.42 at 5 days after ingestion. The corresponding value with the young calf was 2.48 per cent, which indicates increased absorption. It should also be pointed out that with the young animal a larger proportion of the cobalt was found in the blood. In general, the distribution of the cobalt absorbed from the tract paralleled that of the injected cobalt.

Comparisons of the data on swine, rabbits, and cattle show no variations which would indicate a species difference as far as the internal metabolism of cobalt is concerned.

There are two obvious rôles for cobalt as a dietary essential. This element may be a specific essential for the ruminant, acting not on the animal itself, but having a localized function in the rumen contents, or in addition it may have a hematopoietic function essential for all species. The contention that the former represents the major function in the ruminant is supported by indirect evidence such as (a) observations that non-ruminants, horses, for instance, on cobalt-deficient pastures, apparently do not suffer from cobalt deficiency, (b) the fact that cobalt deficiency is not ob-

<sup>1</sup> Ely, R. E., Dunn, K. M., Huffman, C. F., Comar, C. L., and Davis, G. K., unpublished results.

served in young calves until after the rumen has started to function, and (c) the finding that injected cobalt, which the present work has shown does not reach the rumen contents, is relatively inefficient in curing cobalt deficiency. Evidence in support of an essential hematopoietic function of cobalt is lacking, an argument against such a rôle is the consideration that the cobalt requirement of non-ruminants is so small that cobalt deficiency has not been reported with natural diets nor produced on artificial rations. Houk *et al* (7) report that the cobalt requirement of rats is less than 0.03  $\gamma$  per rat per day. Mature cattle remain in normal health on a cobalt intake of 1 mg per day, since the present work indicates that less than 10 per cent of this would be available for internal metabolism, a 500 to 700 pound animal would require not more than 0.1 mg per day for hematopoietic needs. On the other hand, the ability of cobalt to stimulate erythrocyte formation has been established and the present work demonstrates that some dietary cobalt, even though a very small percentage of intake, does reach those tissues concerned in the formation of hemoglobin and red blood cells, namely, the true stomach mucosa, intestinal mucosa, spleen, liver, and red bone marrow.

#### SUMMARY

The tissue distribution of radioactive cobalt injected into the jugular vein of a month-old calf was similar to that previously reported for mature animals. However, a young calf absorbed a greater percentage of orally administered cobalt than did older animals, and the tissue distribution of the cobalt absorbed from the tract paralleled that of the injected cobalt.

Comparisons of similar data on swine, rabbits, and cattle showed no variations which would indicate a species difference in regard to the internal metabolism of cobalt. The present findings support the view that the major function of cobalt in the ruminant is a localized action in the rumen, but do not exclude the possibility of a hematopoietic function, which, if it does exist, is probably catalytic in nature as indicated by the small amount of the element involved.

The writers wish to express appreciation to Dr R. B. Becker, Dr R. S. Glasscock, and Mr P. T. Dix Arnold for making some of the animals available and assisting with the autopsies, and to Mrs Martha Michael for technical assistance.

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## CHROMOGENIC SUBSTRATES

### III *p*-NITROPHENYL SULFATE AS A SUBSTRATE FOR THE ASSAY OF PHENOLSULFATASE ACTIVITY\*

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(Received for publication, June 2, 1947)

This paper describes the use of *p*-nitrophenyl sulfate as a chromogenic substrate for the colorimetric assay of the activity of phenolsulfatase. In neutral or alkaline solution the substrate is colorless, on enzymatic hydrolysis *p*-nitrophenol is liberated, which at alkaline pH develops a yellow color suitable for the quantitative measurement of activity of the enzyme. Advantages which this method holds over earlier procedures include greater simplicity, accuracy, and sensitivity. The procedure is similar in principle to that used in the assay of phosphatase with phenolphthalein phosphate (1) or *p*-nitrophenyl phosphate (2) as substrates for phosphatase action and the method for assay of  $\beta$ -glucuronidase with phenolphthalein  $\beta$ -monoglucuronide (3), in these methods the amount of color due to liberation of indicator from the substrate under standard conditions is a measure of enzymatic activity.

**Previous Methods**—Derrien (4) discovered that an extract of certain shell-fish, *Murex trunculus*, hydrolyzed indoxyl sulfates obtained from human urine with the liberation of indigo in a blue form. Neuberg and Wagner (5) also used this qualitative test to demonstrate sulfatase activity in taka-diastase. This enzymatic activity was studied quantitatively by Neuberg and Kurono (6), who named the enzyme sulfatase. At first they used potassium phenyl sulfate as a substrate and employed the distillation of phenol or the gravimetric determination of barium-precipitated sulfate as indices of sulfatase activity. Later, using sulfate esters of the three isomers of nitrophenol as substrates, Neuberg and Wagner (5) extracted free nitrophenol with ether from the aqueous solution after enzymatic action, the ether was then evaporated and the nitrophenol was weighed. Neuberg and Simon (7), on the basis of specificity, designated the enzyme capable of splitting sulfate esters of phenols as phenolsulfatase. Abbott (8) determined phenolsulfatase activity by measuring colorimetrically the amount of phenol liberated from potassium phenyl sulfate.

**Preparation of Potassium *p*-Nitrophenyl Sulfate**—The following method

\* This investigation was supported by grants from the Albert and Mary Lasker Foundation, Inc., the Sidney and Frances Brody Foundation, Mr. Ben May, and the Committee for Research in Problems of Sex of the National Research Council.

is adapted from Burkhardt and Lapworth (9). Dimethylaniline, 47 ml, and carbon disulfide, 50 ml, are mixed in a 500 ml suction flask and placed in an ice bath under a hood. To the chilled mixture 9.1 ml of chlorosulfonic acid are added dropwise, then 13.9 gm of *p*-nitrophenol are added rapidly and the mixture is stirred for 1 hour before being allowed to stand overnight. 100 ml of 0.4 *N* potassium hydroxide are added, causing the immediate formation of bright yellow crystals. After thorough stirring of the mixture, the flask is placed in a water bath at a temperature of about 80° and most of the carbon disulfide is evaporated *in vacuo*. Excess dimethylaniline is then conveniently separated by centrifugation and the residual yellow mass is dried *in vacuo*. The crude product is recrystallized three to four times in 80 per cent ethyl alcohol.

The recrystallized potassium *p*-nitrophenyl sulfate contained less than 0.06 gm per cent of free *p*-nitrophenol. The dry compound was stored at 0° to minimize decomposition.

#### EXPERIMENTAL

##### *Reagents—*

0.5 *N* acetate buffer, pH 5.8. 63.97 gm of sodium acetate are dissolved in distilled water. 170 ml of 99 per cent glacial acetic acid are added and the mixture is diluted to 1 liter.

0.005 *N* potassium *p*-nitrophenyl sulfate. 0.1285 gm of potassium *p*-nitrophenyl sulfate is dissolved in distilled water and made up to 100 ml.  
1 *N* sodium hydroxide.

*p*-Nitrophenol stock solution. *p*-Nitrophenol (Eastman Kodak Company) is recrystallized twice from aqueous solution and 100 mg are dissolved in 100 ml of water.

Taka-diastase. 1.6 gm of taka-diastase (Parke, Davis and Company) are dissolved in 100 ml of water.

*p*-Nitrophenol Calibration Curve.—From the stock *p*-nitrophenol solution, serial dilutions are made from 0.25 to 10.0 mg per liter. 5 ml of each dilution are pipetted into colorimeter tubes and 5 ml of 1 *N* NaOH are added to each. The readings on the colorimeter (per cent transmission of light) are plotted on semilog paper against micrograms of *p*-nitrophenol in the tube. Distilled water is used as a blank with the scale set at 100 per cent light transmission with a 420 *mμ* filter. An Evelyn photoelectric colorimeter was used throughout. In alkaline solution, maximum color of *p*-nitrophenol was observed between pH 9 and 11 and there was no fading of the intensity of color in 5 hours.

*Method of Assay*.—Clean, uniform, rubber-stoppered colorimeter tubes measuring 170 × 20 mm are used. Each determination is run in duplicate with a single control. 3 ml of acetate buffer, 1 ml of enzyme solution,



and a tiny crystal of thymol are added to each tube. All the tubes are placed in a constant temperature water bath at  $37^{\circ}$  and allowed to equilibrate for 5 minutes. 1 ml. of substrate solution is pipetted at 1 minute intervals into each of the experimental tubes only. Each tube is swirled lightly and stoppered. After incubating for the precise period of 10 hours, 5 ml. of 1 N sodium hydroxide are pipetted into each tube, including the controls. 1 ml. of substrate solution is then added to each control. The tubes are inverted several times and wiped dry of water from the bath. The intensity of color is measured in the Evelyn colorimeter with a 420 m $\mu$  filter with a water blank set at 100 per cent transmission. From the standard calibration curve the content of *p*-nitrophenol is obtained, the average of the duplicate readings minus the control gives the amount of activity expressed in micrograms of the indicator. 1 phenolsulfatase unit is defined as the amount of enzyme which produces the color equivalent to 10  $\gamma$  of *p*-nitrophenol in a volume of 10 ml. in 10 hours at  $37^{\circ}$  in 0.5 N acetate buffer at pH 5.8, the substrate concentration being 0.005 M.

Body fluids such as serum and urine were assayed directly. In preparing tissues, conveniently small pieces were cut from a freshly exsanguinated rat or from a fresh surgical specimen, and weighed on a torsion balance. The tissue was transferred to a glass homogenizer before 5 ml. of ice-cold distilled water were added. After rapid homogenization for 2 to 3 minutes, the suspension was centrifuged for 10 minutes. The supernatant homogenate was then chilled until ready for assay. Sulfatase activity in duplicate samples of the same liver checked within  $\pm 4.5$  per cent (Table I).

*Optimum pH for Activity*—The effect of pH on the rate of hydrolysis of substrate was studied with acetate buffers between pH 4 and 6.6, the normality of acetate buffer was varied between 0.01 and 2 N also. The velocity of sulfatase action was tested likewise with phosphate buffers between pH 6 and 8.12. All pH determinations were made electrometrically. There was greater activity in acetate than in phosphate and the greatest activity occurred at pH 6.12 when the system was buffered with 0.5 N acetic acid-sodium acetate (Fig. 1). Because of greater buffering power this buffer system was used at pH 5.8 in the present experiments.

*Optimum Substrate Concentration*—The maximum velocity of phenolsulfatase activity was found to occur with a substrate concentration of 0.015 M (Fig. 2). A considerably lower concentration of 0.005 M was found to be satisfactory for routine use. The Michaelis and Menten (10) constant was found to be  $0.7 \times 10^{-4}$  M.

*Termination of Enzymatic Activity*—Phenolsulfatase activity is completely inhibited above a pH of about 8. 5 ml. of 1 N sodium hydroxide increase the pH of the incubation mixture from pH 5.8 to a pH of about 10.5.

*Relationship of Activity to Enzyme Concentration and to Time*—A linear

relationship was found between the rate of hydrolysis and enzymatic concentration with serial dilutions of both taka-diastase and liver homogenate

TABLE I  
*Multiple Assays of Sulfatase Activity in Single Rat Liver*

Sample No	Wet weight of tissue	Phenolsulfatase units*
	mg	
1	28.2	7.2
2	39.4	6.75
3	46.8	6.35
4	55.6	6.45
5	41.6	6.6
Average		6.67 ± 0.3

\* Per 1 mg. of wet tissue

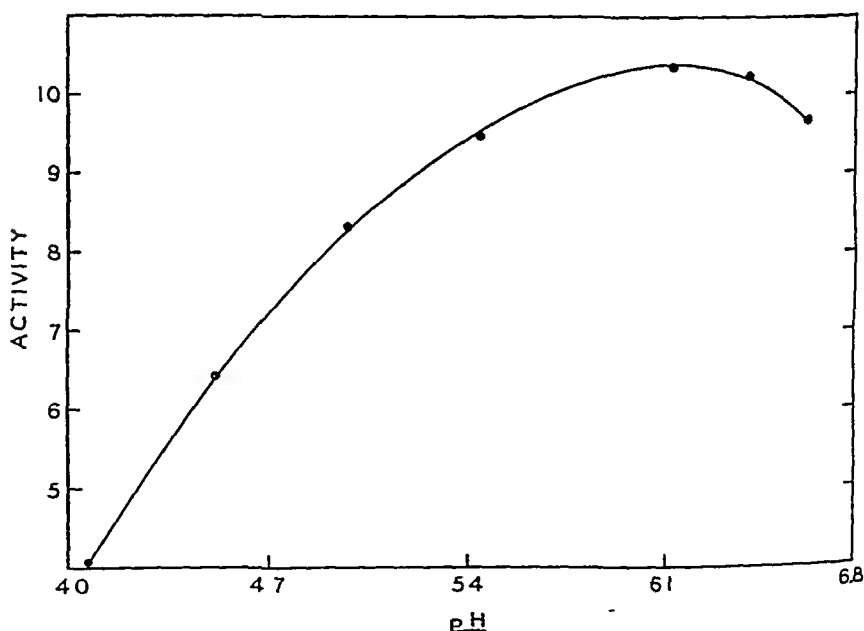


FIG 1 The effect of pH on the activity of the phenolsulfatase in a 1.6 per cent taka-diastase solution in 0.5 N acetate buffer, ordinates, units of sulfatase activity, abscissae, pH

(Fig 3) Experiments with a series of duplicate samples also established linear relationship between activity and periods of time varying from  $\frac{1}{2}$  to 4 hours (Fig 4)

*Inhibition of Bacterial Decomposition*—Although no effect from bacteria was observed, a minute crystal of thymol was added to each incubation mixture to prevent possible bacterial decomposition of substrate. Thymol was found to have no effect upon colorimeter readings either before or after incubation, and was found neither to inhibit nor enhance this enzyme action.

*Accuracy and Sensitivity*—In 61 determinations of the phenolsulfatase activity of tuka diastase solutions a standard deviation in duplicates of

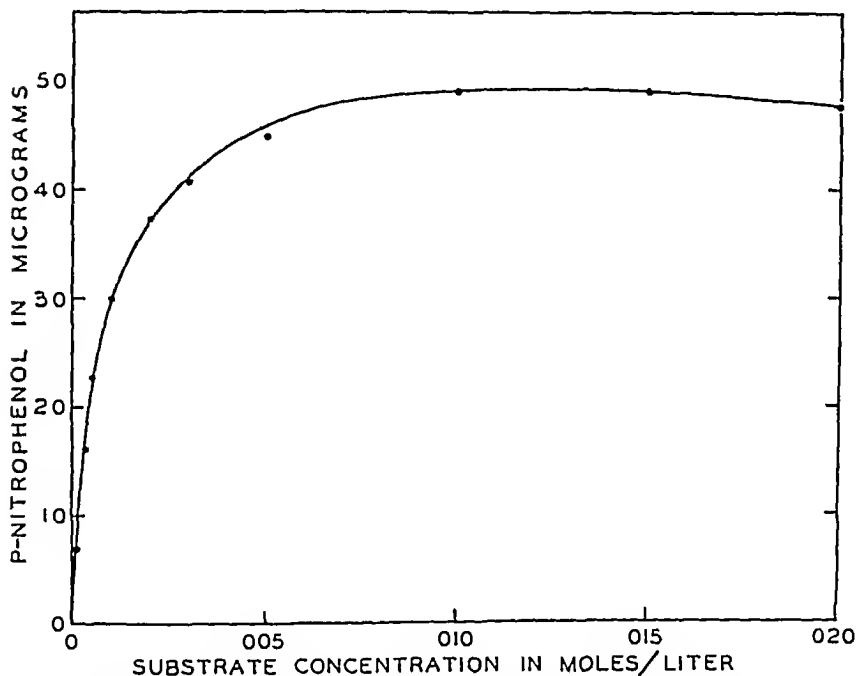


FIG. 2 Effect of substrate concentration on rate of enzymatic hydrolysis of *p*-nitrophenyl sulfate. The system consisted of 3 ml. of 0.5 *N* acetate buffer at pH 5.8, 1.0 ml. of tuka diastase (1.6 per cent) dissolved in water, and 1.0 ml. of *p*-nitrophenyl sulfate in various concentrations. Reaction terminated and color developed with 5 ml. of 1.0 *N* sodium hydroxide after 3 hours incubation.

$\pm 1.10$  per cent was observed. The consistency of assays of multiple samples of an individual rat liver is shown in Table I.

The sensitivity of the method is illustrated by the constant detection of phenolsulfatase in serum with the present method. In twenty-four human sera the range varied from 0.3 to 15.5 units per 1 ml. Abbott (8) did not observe enzymatic activity in serum with potassium phenyl sulfate as a substrate, even after 18 hours incubation.

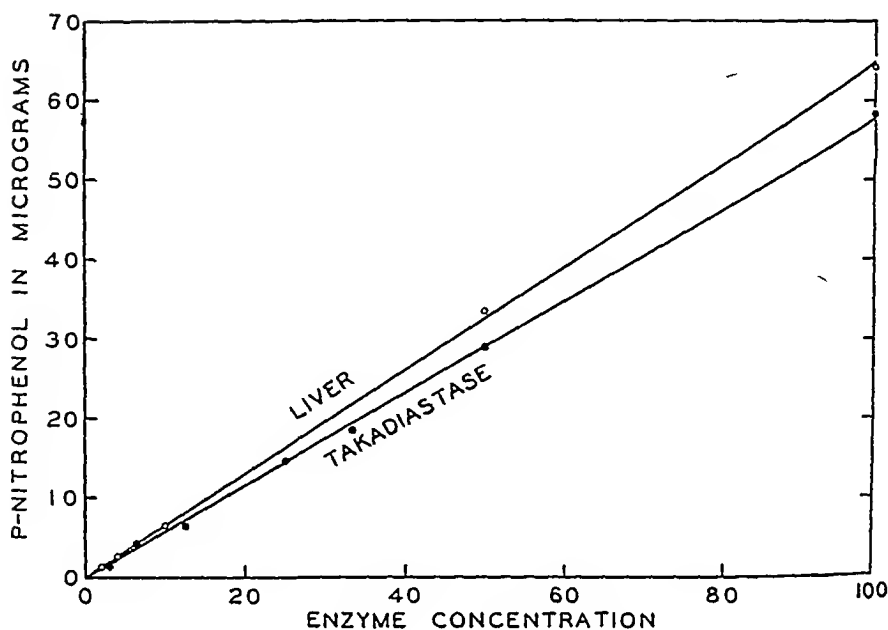


FIG 3 The linear relationship of enzyme concentration and reaction product. The sources of enzymes were 1.6 per cent taka-diastase solution and rat liver homogenate diluted according to the percentage values indicated on the abscissa, the ordinates are micrograms of *p*-nitrophenol liberated in 10 hours.

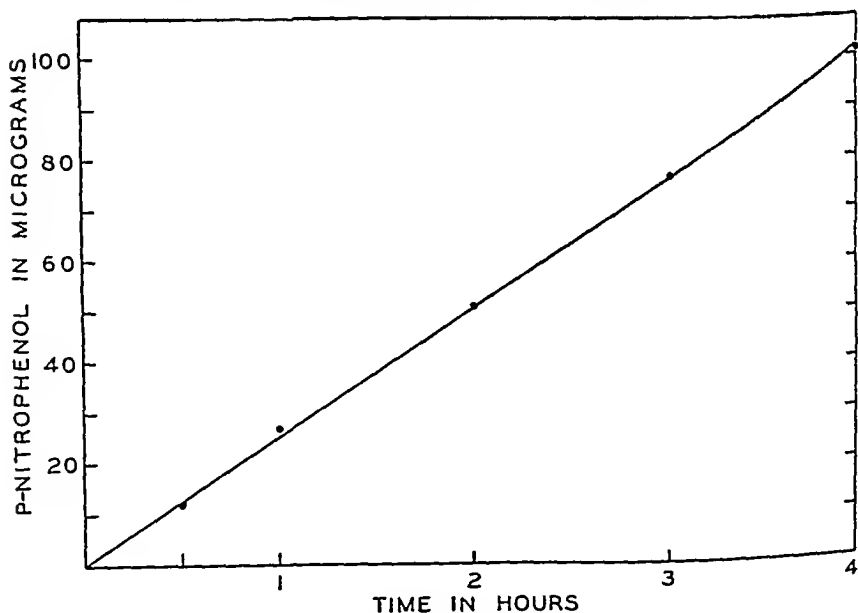


FIG 4 The course of enzymatic hydrolysis of *p*-nitrophenyl sulfate by taka-diastase, 1.6 per cent. The ordinates indicate micrograms of *p*-nitrophenol liberated.

### Results

The results obtained in the assay of homogenates of organs in the rat show (Table II) that relative activity is greatest in liver and the suprarenal gland. In the suprarenal of the dog the greater concentration of sulfatase was in the medulla, in which average values per mg were found to be 1.28 units for medulla and 0.34 unit for cortex.

Human urine always showed sulfatase activity, values of 0.9 to 19.7 units per l. ml. were obtained in twenty-four cases.

Sulfatase was found in neoplasms of the rat. In the transplantable pleomorphic Sarcoma 39 of the rat, phenolsulfatase values of 0.8 to 1.68 units per mg. of tissue were obtained in six cases, in skeletal muscle and

TABLE II  
*Assay of Phenolsulfatase Activity in Organs of Rat*

Organ	No. of rats assayed	Phenolsulfatase units*	
		Range	Mean
Liver	10	3.69-7.96	6.58
Suprarenal	1	3.08-5.56	4.23
Kidney	1	1.71-5.60	3.63
Spleen	3	1.31-2.66	1.89
Lymph node	3	1.52-1.71	1.61
Lung	3	0.37-1.45	0.89
Thyroid	3	0.16-0.94	0.48
Prostate	3	0.30-0.88	0.47
Testis	3	0.22-0.51	0.40
Heart	3	0.05-0.75	0.39
Brain	3	0.27-0.51	0.37
Skeletal muscle	3	0.09-0.28	0.17

\* Per 1 mg. of wet tissue

connective tissue, believed to be the tissues of origin of this cancer, the mean values were 0.17 unit and 0.015 unit respectively. In three spindle cell sarcomas in the rat induced by subcutaneous injection of 20-methylcholanthrene the range was found to be 0.59 to 0.67 unit per gm.

### SUMMARY

1. Colorimetric assay of phenolsulfatase activity with *p*-nitrophenyl sulfate as a substrate presents advantages of simplicity, sensitivity, and accuracy.

2. A rectilinear relationship exists between enzymatic activity and both time and enzyme concentrations. The optimum activity of the enzyme occurs at pH 6.12 in 0.5 *N* acetate buffer at 37°. The Michaelis and Menten constant was found to be 0.0007 *M*.

3 Human serum and urine constantly contained small amounts of sulfatase. Next to liver, the greatest sulfatase activity was found in the suprarenal gland, with greater concentration in the medulla than in the cortex. Sarcomas of the rat contained considerable activity.

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# FURTHER STUDIES ON LIPIDE STIMULATION OF *LACTOBACILLUS CASEI*

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(Received for publication, May 2, 1947)

Oleic acid stimulation of *Lactobacillus casei* was discussed in the most recent paper of this series (1). At that time, the formulation of hypotheses regarding the precise manner of function of oleic acid was hampered because of uncertainties regarding the degree to which the media used were truly biotin-free. A further hindrance lay in the dearth of information concerning the behavior of *L. casei* in the presence of other surface-active materials. Lastly, there was an evident need for fundamental experiments to elucidate the nature of the stimulation phenomenon. In accordance with these needs, the study of lipide stimulation of *L. casei* has been continued along the three lines indicated: (a) attempts to secure a biotin-free medium in which to observe stimulation, (b) experiments with synthetic detergents of several types, and (c) a study of oxidation-reduction potentials in normal and stimulated cultures.

## EXPERIMENTAL

**General Procedure**—The routine methods of preparing and sterilizing media, making inoculations, incubating, and determining acidity or turbidity have been described in the previous paper (1). The terminology described there for discussing results obtained with normal and stimulated cultures will be used here also. To reiterate, standard tubes are referred to as Tubes S, with the micromicrograms of biotin given as a subscript, e.g., Tubes S<sub>1000</sub> for the standard tubes containing 1000 micromicrograms of biotin in 10 ml of medium. Tubes containing 100  $\gamma$  of oleic acid are referred to as Tubes OA with a subscript to indicate micromicrograms of biotin, e.g., Tubes OA<sub>0</sub> for tubes containing 100  $\gamma$  of oleic acid but no added biotin per 10 ml of medium. The oleic acid was the same as that used in the experiments described previously and was added to the medium in the same fashion.

**Experiments with Biotin-Free Media**—In the effort to observe the effects of lipides on *Lactobacillus casei* in truly biotin-free media, three types of media were examined: (a) media in which casein hydrolysate was replaced by mixtures of pure amino acids, (b) amino acid media in which the non-synthetic amino acids, purines, and pyrimidines were treated with peroxide,

and (c) standard casein hydrolysate medium mentioned above as well as amino acid medium to both of which was added avidin in the form of egg white solutions

*Experiments with Amino Acid Media*—Two amino acid mixtures were compared with casein hydrolysate as to oleic acid stimulation. The first was based on the studies of Hutchings and Peterson (2) and has been designated here as Medium A, and the second, Medium B, formulated according to the amino acid requirements of *Lactobacillus casei* as described by Hac, Snell, and Williams (3). With the exception of L-proline, which is not included in the list of Hutchings and Peterson, the two media contained the same amino acids in different proportions, Medium B being much higher in aspartic and glutamic acids. Attempts to omit cystine, tyrosine, isoleucine, and phenylalanine (since these have been designated as non-essential by Hac, Snell, and Williams) resulted in inferior growth in Tubes S<sub>1000</sub>. Both media when complete with all the amino acids required would support growth, the ranges differing, although neither so well as the casein hydrolysate medium. The data are presented in Table I.

When the casein hydrolysate medium was replaced by amino acid mixtures, as may be seen from Table I, oleic acid stimulation was not prevented, although it occurred to a lesser extent on Medium B (compared with casein hydrolysate) and very much less on Medium A. When the amino acid cultures were permitted to grow 96 hours, however, as in Experiment 3, the extent of stimulation was greatly increased. Such a lag has been shown before to be typical of oleic acid stimulation.

*Synthetic Media with Peroxide-Treated Amino Acids*—Since it proved impossible to prevent stimulation by replacing casein hydrolysate with amino acids, peroxide treatment of the amino acids was tried as a means of reducing residual biotin, it being at the time assumed that stimulation was dependent on the presence of some biotin. Ideally, a medium to be biotin-free should contain only synthetic amino acids, purines, and pyrimidines. Quite a number of these essential constituents are at present available only as products extracted from natural materials. In so far as possible, however, a medium was made in which the constituents were synthetic, the natural materials then being subjected to peroxide treatment. The peroxide treatment of solutions of amino acids and nitrogen bases was conducted in the manner described for yeast extract by Shull, Hutchings, and Peterson (4). Since it was assumed that any traces of biotin would be in only the natural amino acids, no DL products were so treated. Correspondence with the various manufacturers confirmed this assumption. Instead of oleic acid, one of the non-ionic detergents to be described below, Nopalcol 6-O, was used, since this material had in the meantime proved to be a much more powerful stimulator than oleic acid. Tubes containing



Nopalcol were labeled Tubes N with micromicrograms of biotin given in the subscript. As a repeated check on experiments conducted in the past with pantothenic acid-free medium, riboflavin-free medium, and biotin-free medium, the same peroxide-treated L-immunoids, purines, and pyrimidines, all in one solution, were used along with the rest of the DL acids

TABLE I  
*Oleic Acid Stimulation in Casein Hydrolysate Medium Compared with Two Synthetic Media*

Experiment No.	Medium	0.1 acid per tube				
		Tube S0S	Tube S <sub>0</sub>	Tube S <sub>1000</sub>	Tube OA <sub>0</sub>	Tube OA <sub>1000</sub>
		ml	ml	ml	ml	ml
1	Casein hydrolysate		2 12	16 35	7 62	8 33
	Medium A		2 39	13 63	2 55	3 31
	" B		2 01	15 41	3 84	4 47
2	Casein hydrolysate		2 70	17 23	9 83	14 62
	Medium A		2 23	15 99	7 09	8 77
3†	" "	1 66	2 54	16 74	3 77	11 10
	" B	1 63	2 89	15 93	8 82	9 03

\* Uninoculated

† Experiment extended to 96 hours

TABLE II  
*Lipide Stimulation from Nopalcol 6 O in Three Peroxide Treated Media*

Medium	0.1 N acid per tube				
	Tube S <sub>0</sub> *	Tube S <sub>0</sub>	Tube S <sub>max</sub> †	Tube N <sub>0</sub> †	Tube N <sub>max</sub> ††
	ml	ml	ml	ml	ml
Biotin free	2 14	2 85	10 12	12 48	12 41
Pantothenic acid free	2 45	3 49	8 90	3 15	9 16
Riboflavin free	2 40	2 51	10 20	2 44	12 15

\* Uninoculated

† Maximum values on standard curves were as follows: biotin, 1000 micromicrograms per tube, calcium pantothenate, 0.20  $\gamma$  per tube, and riboflavin, 0.20  $\gamma$  per tube

†† Tubes N contained 10.0 mg of Nopalcol 6 O per tube

and other medium constituents as a basis for three such media. These three media were made from portions of a base mixture, containing everything to be included except vitamins, these being added separately. The data obtained are shown in Table II.

Tubes containing the maximum concentration of vitamin used for the

standard curve are indicated by the subscript  $\max$ . It is immediately evident from these data that the growth range of *Lactobacillus casei* in all media was much smaller than that usually obtained (compare Tubes  $S_{1000}$  in Tables I and III). Undoubtedly something was unintentionally affected by the peroxide treatment. This state of affairs, however, in no way interfered with the objective of the experiment, *ie*, to determine whether or not lipid stimulation could be obtained on such media. As has been observed in the past, stimulation was obtained in the zero tubes with the biotin-free medium, but none was found with the riboflavin-free or pantothenic acid-free medium. In all three media, Tubes  $N_{\max}$  produced greater growth than did the corresponding Tubes  $S_{\max}$ . The significance of this finding will be discussed below in connection with other experiments with synthetic detergents.

*Avidin Experiments*—Since, according to Eakin, Snell, and Williams (5), 1 ml of raw egg white will inactivate 0.9 to 1.2  $\gamma$  of biotin, the egg white was diluted 1:800 with sterile 0.2 per cent ammonium sulfate solution, 1 ml of such a dilution being capable of binding 1000 micromicrograms of biotin. Dilute ammonium sulfate solution was used instead of distilled water because solution of the protein in distilled water did not give satisfactory dispersion, the egg white becoming opalescent and tending to remain in discrete globules rather than to diffuse into the water. 1 ml of the solution of protein in ammonium sulfate solution was pipetted aseptically into each tube. Sterilization of the protein solution by filtration through a sterile Chamberland-Pasteur filter candle produced an inactive filtrate. Landy *et al* have reported similar findings (6). No irregular results were observed as a consequence of using non-sterile egg white solution. All glassware and media were sterilized in the usual manner, the casein hydrolysate medium was the same as that used in the above experiments, and Medium B was used for the amino acid medium. All tubes containing the added egg white were set up in quadruplicate and half the tubes steamed for 15 minutes at 100° to serve as controls. The results obtained with Tubes  $S_0$ ,  $S_{1000}$ ,  $OA_0$ , and  $OA_{1000}$  may be seen in Table III.

In the presence of egg white, biotin was bound in all tubes of the standard curve, although only Tubes  $S_0$  and Tubes  $S_{1000}$  have been presented in Table III. The slight differences obtained in every case between Tubes  $S_0$  uninoculated and Tubes  $S_0$  and Tubes  $S_{1000}$  containing egg white represent growth due possibly to the presence of biotin, unremovable from the cells of the inoculum, which may in some way be protected from avidin by being already bound to protein. The principal finding is that the avidin, although able to bind all the biotin in the medium, did not interfere with stimulation of growth by oleic acid. Even greater growth was obtained in Tubes  $OA$  which contained steamed egg white. This increase, which

was also evident in steamed Tubes  $S_0$ , might be due to the release of biotin from the egg white plus some stimulating effect from lipides released from egg white. The standard curves of the steamed standard (not given in entirety) showed increased growth in the tubes of lower biotin concentration when compared with the corresponding tubes of the standard containing no egg white. The higher concentrations were practically identical with the standard containing no egg white. This shift indicates a stimulation rather than a release of biotin, since biotin release would produce uniform increases in all tubes. Cognizance was taken of the possibility that the presence of oleic acid in the medium at the time of addition of

TABLE III  
*Effect of Avidin on Oleic Acid Stimulation of Lactobacillus casei*

Tubes	0.1 N acid per tube					
	Casein hydrolysate medium			Amino acid medium		
	Without egg white	Egg white added	Egg white added and steamed	Without egg white	Egg white added	Egg white added and steamed
	ml	ml	ml	ml	ml	ml
$S_0^*$	1.33	1.31	1.26	2.00	2.01	2.08
$S_0$	2.16	2.03	3.19	2.58	2.93	4.44
$S_{1000}$	16.27	2.00	16.35	14.50	3.21	15.61
$OA_0$	7.25	10.83	11.10	5.47†	9.63†	10.01†
				4.22†	8.20†	8.83†
$OA_{1000}$	11.32	11.10	15.37	7.12†	10.84†	11.48†
				6.40†	8.71†	11.85†

\* Uninoculated

† Egg white solution was added as the last component to the tubes 30 minutes before inoculation

‡ Oleic acid emulsion was added as the last component to the tubes 30 minutes after addition of egg white solution and immediately before inoculation

egg white might interfere with binding of biotin by avidin, therefore, in one set of tubes the oleic acid emulsion, previously sterilized, was added to the medium and avidin after a 30 minute incubation period. In all cases but one, as may be seen from Table III, this procedure resulted in a slightly lowered acid production but did not appreciably interfere with oleic acid stimulation. The slight reduction in stimulation observed might be attributable to some physical factor, such as slight breakdown of emulsion on autoclaving in sterile water as compared with the usual highly buffered medium.

As was the case with the casein hydrolysate medium, the presence of avidin in the amino acid medium did not prevent stimulation from oleic acid, although it effectively bound biotin in the standard tubes. It should

be noted that at the end of the 72 hour growth period, Tubes  $S_{1000}$  on the amino acid medium did not produce so much acid as those on the casein hydrolysate medium. This occurrence has been mentioned before in connection with the synthetic media described in the preceding paragraphs. In the steamed Tubes  $S_{1000}$  of the amino acid medium an appreciable increment of growth occurred, indicating the release of some substance conducive to acid production. As with the casein hydrolysate medium, however, the increase in the steamed standard tubes was not uniform throughout the curve, but was greatest in the low biotin tubes.

*Synthetic Detergents*—The synthetic detergents examined were obtained from the National Oil Products Company (Nopco), Harrison, New Jersey, and the Atlas Powder Company, Wilmington, Delaware. A list of the detergents with information as to their chemical nature and stimulating effect is given in Table IV. The detergents were added to media as a water dispersion whenever possible, and in some cases as an alcohol dispersion. Detergents which proved difficult to disperse were homogenized with the solvent in the Waring blender, and the resulting solution diluted and pipetted as necessary. In Table V is shown the effect of increasing concentration of the most effective detergents on acid production in tubes containing no biotin. These data when plotted showed a surprising linearity. Nearly all of the detergents examined proved stimulatory even at high concentration. The extent of stimulation produced at these concentrations, however, was considered remarkable. Oleic acid, which is surface-active and anionic, has been shown (1) to produce its maximum stimulation at a concentration of 400  $\gamma$  per tube, above which it becomes increasingly inhibiting. Of the few anionic detergents examined here as well as the one effective cationic detergent, all those which proved stimulatory showed a maximum effective concentration above which stimulation decreased. It should be emphasized that these experiments were designed to reveal stimulation and not inhibition, since only the former would be detected in tubes containing no biotin. Had inhibition rather than stimulation been the primary interest, the inhibition tests with Tubes  $S_{1000}$  rather than Tubes  $S_0$  for standards would have been made.

Of the non-ionic detergents, Table IV shows Nopalcols 6-O and 10-O, Tweens 20, 60, and 80, Spans 60, 80, and 85, G-1226, and Arlacel C to be the most stimulating. Of these compounds, the majority are oleic acid derivatives and quite likely traces of oleic acid derivatives are to be found in the others. In concentrations as great as 50 mg. per tube, Tweens 20, 60, and 80, Arlacel C, Spans 60, 80, and 85, Nopalcols 6-O and 10-O, and G-1226 were still able to effect approximately 100 per cent conversion of glucose to acid. Table IV indicates that the concentration necessary to

obtaining maximum acid production observed for the most effective detergents was between 1500 and 5000  $\gamma$  per tube. In many cases the increments obtained in raising the concentration above 1500  $\gamma$  were of the magnitude of

TABLE IV  
*Synthetic Detergents and Their Effects in Stimulating Lactobacillus casei*

Name	Manufacturer	Chemical nature	Point of maximum stimulation in biotin free tubes	
			Detergent per tube	0.1 N acid per tube
			$\gamma$	ml
Nopeocastor	Nopeco	Anionic sulfated castor oil	250	3.93
Syntergent K	"	Cationic substituted lauric amine	250	5.74
Nopaleol 6 O	"	Non ionic oleate	1,500	23.56
" 6 L	"	" laurate	200	8.87
" 6 R	"	" ricinoleate	5,000	8.37
" 6 S	"	" stearate	5,000	14.13
" 10 O	"	" oleate	5,000	22.54
Nopco 1255	"	Anionic sulfated butyl oleate	No effect	
" 2173 B	"	Cationic oleic amine sulfate	" "	
Tween 20	Atlas Powder	Non ionic laurate	50,000	23.41
" 40	" "	" palmitate	40,000	20.43
" 60	" "	" stearate	50,000	23.27
" 61	" "	" "	50,000	12.70
" 80	" "	" oleate	2,000	22.61
" 81	" "	" "	40,000	19.79
" 85	" "	" trioleate	10,000	22.09
Span 20	" "	" laurate	750	5.27
" 10	" "	" palmitate	50,000	17.93
" 60	" "	" stearate	50,000	22.60
" 80	" "	" monooleate	3,500	23.16
" 85	" "	" trioleate	50,000	21.38
Arlacel C	" "	" sorbitan sesquileate	5,000	23.16
G-1226	" "	Non ionic ether ester	2,500	21.50
G-7596	" "	" laurate	50,000	9.43

only 1 or 2 ml of 0.1 N acid, a leveling off effect occurring at approximately that concentration. Although the data are not presented in a table, it should be mentioned that the effect of pH observed with oleic acid (1) was not found to be the case with these non-ionic detergents. Decreasing the

$N_2$  simultaneously. The curve obtained for Flask  $S_{1000}$  closely resembles those obtained by Hewitt for hemolytic streptococci. According to McLeod's classification of organisms (8), *Lactobacillus casei* would fall into the class of peroxide producers, devoid of catalase, and relatively insensitive to peroxide. Pneumococci and certain streptococci are also members of this class. The drop in potential following a period of oxygen uptake by the medium is due to the reducing activities of the bacteria and represents the period of their logarithmic growth. The slow rise in potential following the drop is caused by the accumulation of peroxide in the medium, according to Hewitt. It is at once obvious that the presence of Nopalcol resulted in the culture's attaining a lower potential than possible with biotin and indicates the stimulation of reducing activities. The lag period observed in acid and cell production in the presence of oleic acid (Flask  $OA_0$ ) is illustrated well in terms of oxidation-reduction potential.

#### DISCUSSION

In considering first the experiments designed to produce a biotin-free medium, it appears more and more evident that *Lactobacillus casei* can be made to grow in the absence of biotin provided a suitable detergent is present in the medium. When a sufficient variety of synthesized amino acids, purines, and pyrimidines is manufactured so that one can designate a medium as biotin-free without making the currently necessary mental reservations, at least this phase of the question can be settled. It is at any rate obvious that substituting an amino acid mixture for casein hydrolysate, or, further, treating those amino acids with peroxide prior to their use, or even adding more than enough avidin to bind all free biotin in the medium, does not prevent lipide stimulation of *Lactobacillus casei*.

The experiments on detergents reported here represent generally further studies of the type initiated by Baker, Harrison, and Miller (9-11). Most of the fatty acids and other fat-soluble compounds investigated by others have been studied principally from the standpoint of their inhibitory and not their stimulatory effects. The most extensive work to date on bacterial stimulation by lipides is that of Dubos and Davis, working principally with *Mycobacterium tuberculosis* (12-16). The growth of this organism was enhanced by a number of long chain fatty acids, provided the acids were esterified or bound in some way to serum albumin. Lacking one of these two treatments, the fatty acids proved inhibitory. These findings suggest that the lag period observed in oleic acid stimulation of *Lactobacillus casei* might be due to a slow binding of the fatty acid by protein produced by or present as a part of the bacteria. The fact that oleic acid stimulation was obtained to a greater extent on Medium B than Medium A suggests that the overcoming of the lag period was dependent upon a synthesis. Dubos and Davis tested various ones of the Spans and Tweens and found in general

that the oleates and ricinoleates were stimulatory, laurates inhibitory, and stearates of variable effect. As is shown in Table IV, no such categories of effect were observed with *Lactobacillus casei*, although the least effective compound of the group, Span 20, is a laurate.

The experimental determination of oxidation-reduction potentials in the various media studied corroborated the results of acidimetric and turbidimetric investigation. Nopalcol is an oleate and hence, along with oleic acid, would be expected according to the studies of Lepper and Martin (17) to promote reduction of the oxidation-reduction potential of the medium. Part of the effect of long chain unsaturated fatty acids on bacteria is undoubtedly due to absorption of oxygen by the double bonds of the hydrocarbon chains. Lepper and Martin showed it to be the case in enhancement of growth of anaerobes by addition of cooked flesh to anaerobic preparations. Bergstrom, Theorell, and Davide (18) have shown unsaturated fatty acids to be bactericidal to *Mycobacterium tuberculosis* by preventing oxygen uptake by the bacteria. The effect of these fatty acids upon *Lactobacillus casei*, however, cannot be explained in such a fashion, since the organism is a facultative anaerobe and furthermore appears to be stimulated by saturated compounds as well as unsaturated.

An interpretation of the results of experiments such as these hinges upon a satisfactory explanation for the stimulation phenomenon. The bacterial cell cytoplasmic membrane, known to be lipoprotein (19, 20), appears to be the site of the effects in question. The importance of surface effects in such systems is well recognized (21). Franke and Schillinger (22) in commenting on bacterial inhibition by fatty acids have stated that bacterial metabolism is largely influenced by the permeability of the lipid cell membrane. Even more recently, Hotchkiss (23) has presented experimental evidence to show that bacterial membrane permeabilities do change in the presence of detergents, that the membrane is altered to the extent that vital cell contents diffuse into the surrounding medium, and that the cell subsequently undergoes lysis. Stimulation is probably a manifestation of a similar surface effect. If the cell were wetted (by a detergent) just sufficiently to enable it to make better contact with the nutrients of the environment, the cell should grow more rapidly by obtaining its substrate more readily. If, however, the agent producing these changes by virtue of ionic charge or particular molecular configuration were adsorbed in great quantity on disarranged specific vital structures, then the cytoplasmic membrane might be so altered that the effects observed by Hotchkiss would result. Extension of these ideas should help to clarify differences observed in the action of anionic, cationic, and non-ionic detergents on Gram-positive and Gram-negative organisms. Some suggestions in that direction have been made already (24), in addition to ideas presented previously in this paper.

The place of biotin in this picture of surface effect, membrane permeabil-

ity, and the like is debatable. Lipide stimulation occurs, so far as this laboratory has been able to show, in the absence of biotin. On the other hand, it is easy to reduce the pantothenic acid or riboflavin content of a medium until lipide stimulation is completely blocked. The inference is that biotin is not a component of an enzyme system as are these two vitamins, but exerts its influence upon cell surface characteristics and can be dispensed with in the presence of the proper surface-active compound. This idea is not in conflict with the few known facts about biotin function, though it differs from the theories that have been proposed (25, 26). The action of biotin on tissue (27-29) may be due to an effect on cell permeability or to a type of surface alteration. The structure of biotin with its hydrophilic and hydrophobic parts as well as its rapid complex formation with avidin lends weight to such a hypothesis. Further pursuit of these ideas must, of course, await acquisition of additional experimental data.

#### SUMMARY

*Lactobacillus casei* was stimulated by lipides in biotin-free media in which casein hydrolysate was replaced with either (a) amino acids or (b) amino acids, purines, and pyrimidines previously peroxide-treated. The addition of avidin sufficient to bind 1000 micromicrograms of biotin did not prevent lipide stimulation in tubes containing no added biotin.

Various synthetic detergents were examined for stimulatory effect with *Lactobacillus casei*. In general, non-ionic detergents proved stimulatory, many sufficiently so to enable the bacteria to convert glucose into acid with approximately 100 per cent efficiency, even in the absence of biotin. The most stimulatory detergents were oleates.

Oxidation-reduction potentials were determined in growing cultures containing (a) standard biotin or (b) oleic acid in the absence of biotin or (c) a non-ionic detergent in the absence of biotin. The results obtained corroborated acidimetric and turbidimetric data.

On the basis of the experimental data presented and through interpretation of the work of others, it was postulated that biotin functions as a cell permeability factor and can be replaced by the proper lipides.

The authors express their indebtedness to the National Oil Products Company and the Atlas Powder Company for generous gifts of their products.

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## LETTERS TO THE EDITORS

### BIOTIN AND CARBON DIOXIDE FIXATION IN LIVER\*

Sirs

Burk and Winzler<sup>1</sup> suggested that biotin might function as a coenzyme of CO<sub>2</sub> transfer. Koser *et al.*<sup>2</sup> reported that aspartic acid stimulates the

Age	Diet	'Malic' enzyme		Dehydrogenases				Biotin per gm acetone powder
		TPN reduction	Oxalacetate decarboxylation	Lactic	Malic	Isocitric	Glutamic	
days								$\gamma$
30	Normal* (3)	2 20	2 40	178	149	9 5	12 9	3 70
	No biotin (6)	0 96	1 15	245	173	12 3	19 2	0 78
44	Normal (1)	1 83	3 36	282	184	8 5		4 50
	No biotin (1)	0 75	1 79	254	164	12 7		
	Low biotin† (2)	0 64	1 90	302	207	15 1		0 68
	" folic‡ (2)	1 50	2 83	254	180	14 2		5 65
50	Normal (1)	1 59	2 89	313	203	23 2	4 4	6 15
	Low biotin (2)	0 91	1 83	474	235	29 8	6 3	0 41
	" folic (1)	2 34	3 79	366	191	26 4	3 7	4 28

Aqueous extracts of acetone dried turkey livers precipitated three times with ammonium sulfate (80 per cent saturation). Dissolved precipitates dialyzed against 0.02 M phosphate buffer, pH 7.4. Enzyme activities expressed as units per mg of protein. Oxalacetic decarboxylation determined manometrically, all other activities spectrophotometrically. Figures in parentheses, number of livers pooled in each case. Biotin assayed with *L. arabinosus* (Wright, L. D., Biological symposia, Lancaster, 12, 290 (1947)). Autoclaving with 2.0 N H<sub>2</sub>SO<sub>4</sub> at 18 pounds for 1 hour used to liberate biotin from combination.

\* Purified diet (T-15) with vitamin supplements (Jukes, T. H., Stokstad, E. L. R., and Belt, M., *J. Nutr.*, 33, 1 (1947)) including biotin, 200  $\gamma$  per kilo.

† 50  $\gamma$  of biotin per kilo of diet.

‡ 200  $\gamma$  of pteroylglutamic acid per kilo of diet.

\* Supported by grants from the United States Public Health Service, the American Cancer Society (recommended by the Committee on Growth of the National Research Council), the Rockefeller Foundation, and the American Philosophical Society.

<sup>1</sup> Burk, D., and Winzler, R. J., *Science*, 97, 57 (1943).

<sup>2</sup> Koser, S. S., Wright, M. H., and Dorfman, A., *Proc. Soc. Exp. Biol. and Med.*, 51, 204 (1942).

growth of *Torula cremoris* in the absence of biotin and suggested that biotin might be concerned in the synthesis of aspartate. More direct evidence for this was recently obtained by Stokes *et al*<sup>3</sup> with bacteria. Since biotin was not concerned with transamination,<sup>3</sup> it appeared to us that it might be involved in the synthesis of dicarboxylic acids by CO<sub>2</sub> fixation.<sup>4</sup>

The isolation from pigeon liver of a TPN-specific enzyme which catalyzes the reversible conversion of *l*-malate to pyruvate and CO<sub>2</sub> has been reported.<sup>5</sup> Further work suggests that this enzyme also catalyzes the decarboxylation of oxalacetate to pyruvate and CO<sub>2</sub>.

The liver enzyme is markedly decreased in biotin-deficient turkeys, as shown in the table. Whereas both oxidative decarboxylation of malate and decarboxylation of oxalacetate are decreased, the activity of several other enzymes is within the normal range. Furthermore, the "male" enzyme is normal in the livers of folic acid-deficient turkeys. This indicates that the changes in biotin deficiency are specific and are not associated with general malnutrition.

Since biotin is not found in the purified enzyme, it is not clear whether the vitamin participates in its synthesis or is present as a derivative which does not stimulate the growth of *L. arabinosus*. Further work to elucidate these points is in progress.

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Received for publication, July 17, 1947

<sup>3</sup> Stokes, J. L., Larsen, A., and Gunness, M., *J. Biol. Chem.*, **167**, 613 (1947), *Proc. Soc. Am. Bact.*, **47**, 19 (1947).

<sup>4</sup> Using C<sup>14</sup> H. A. Lardy *et al.* (personal communication) have found a marked effect of biotin on the fixation of CO<sub>2</sub> by various bacteria.

<sup>5</sup> Ochoa, S., Mehler, A., and Kornberg, A., *J. Biol. Chem.*, **167**, 871 (1947).

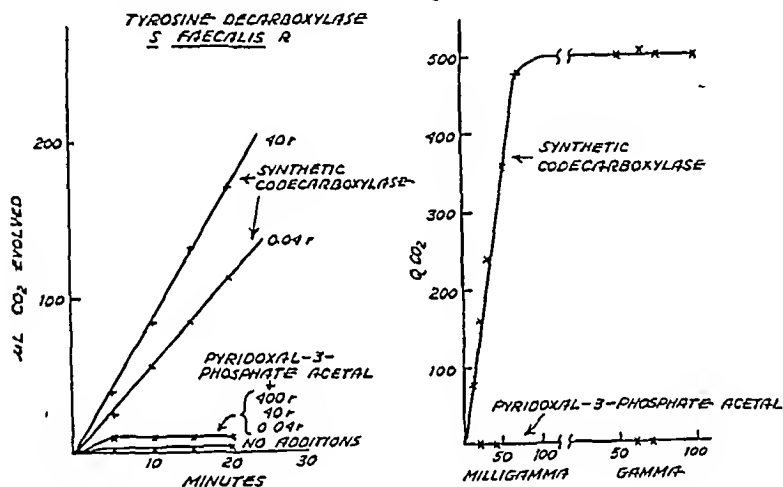
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# CODECARBOXYLASE NOT THE 3-PHOSPHATE OF PYRIDOXAL

Sirs

Reports that synthetic pyridoxal-3-phosphate, in the form of its acetal, possesses codecarboxylase activity<sup>1 2</sup> has prompted us to call attention to evidence to the contrary and to present new data indicating the lack of activity

From published spectral data<sup>3</sup> on this coenzyme and its comparison to the data of Harris, Webb, and Folkers<sup>4</sup> on the behavior of substituted



$\beta$ -pyridones, synthetic pyridoxal phosphate (codecarboxylase) cannot be substituted in the 3 position. Papers by Heyl, Harris, and Folkers<sup>5</sup> and Gunsalus and Umbreit<sup>6</sup> presented further evidence of the structure and eliminated the possibility that codecarboxylase is the 3-phosphate of pyridoxal. The absence of the chlorimide coupling reaction in the co-

<sup>1</sup> Karrer, P, and Viscontini, M, *Helv chim acta*, 30, 52 (1947)

<sup>2</sup> Karrer, P, and Viscontini, M, *Helv chim acta*, 30, 524 (1947)

<sup>3</sup> Gunsalus, I C, Umbreit, W W, Bellamy, W D, and Foust, C E, *J Biol Chem*, 161, 743 (1945)

<sup>4</sup> Harris, S A, Webb, T J, and Folkers, K, *J Am Chem Soc*, 62, 3198-3203 (1940)

<sup>5</sup> Heyl, D, Harris, S A, and Folkers, K, Abstracts, American Chemical Society, 110th meeting, Chicago, 35B (1946)

<sup>6</sup> Gunsalus, I C, and Umbreit, W W, Abstracts, American Chemical Society, 110th meeting, Chicago, 34B (1946)

enzyme is not specific for a linkage of the phenol group, since other substitutions also block this reaction<sup>7</sup>

The activity of synthetic pyridoxal phosphate for the tyrosine decarboxylase apoenzyme is shown in the figure, together with evidence for the lack of activity of the 3-phosphate,<sup>8</sup> prepared and characterized by Heyl, Harris, and Folkers<sup>5</sup>

The essential difference between our experiments and those of Karrer and Viscontini<sup>1</sup> is in the enzyme preparations used. Their tyrosine decarboxylase preparation at a level of 20 mg of dried bacterial cells released about 50 microliters of CO<sub>2</sub> per hour, and on stimulation the rate was increased to 100 microliters per hour. The data in the figure were obtained with 1 mg of dried cell preparation per cup,<sup>9</sup> no CO<sub>2</sub> was released in the absence of coenzyme, and the stimulated rate was 500 microliters of CO<sub>2</sub> per hour. The half maximum rate was reached with 40 mg of the barium salt of the synthetic coenzyme, whereas Karrer and Viscontini<sup>1</sup> used a thousand times this amount of barium pyridoxal-3-phosphate acetal to show traces of activity.

In view of the higher concentration of codecarboxylase required for the glutamic-aspartic transaminase ( $K = 1.5 \times 10^{-6}$ )<sup>10</sup> as compared with the amino acid decarboxylases ( $K = 1.6 \times 10^{-8}$ ) (see the figure), and in view of the limited activity of Karrer and Viscontini's preparation for the decarboxylases<sup>1,2</sup> the lack of activity of their preparation for the transaminase<sup>11</sup> is not surprising.

On the basis of the evidence presented, it is concluded that the pyridoxal 3-phosphate does not possess codecarboxylase activity as reported by Karrer and Viscontini<sup>1,2</sup>

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Received for publication, July 10, 1947

<sup>7</sup> Stiller, E. T., Kercsztesy, J. C., and Stevens, J. R., *J. Am. Chem. Soc.*, **61**, 1237 (1939).

<sup>8</sup> Kindly supplied by the Research Laboratories of Merck and Company, Inc.

<sup>9</sup> Umbreit, W. W., Bellamy, W. D., and Gunsalus, I. C., *Arch. Biochem.*, **7**, 185 (1945).

<sup>10</sup> O'Kane, D. E., and Gunsalus, I. C., *J. Biol. Chem.*, in press.

<sup>11</sup> Karrer, P., and Viscontini, M., *Helv. chim. acta*, **30**, 528 (1947).

# APPARENT SPECIFIC INHIBITION OF PLANT ACETYLESTERASE BY DIISOPROPYL FLUOROPHOSPHATE\*

Sirs

Diisopropyl fluorophosphate (DFP) and hexaethyl tetraphosphate (HETP) have been shown to be remarkably potent inhibitors of cholinesterase both *in vitro* and *in vivo*<sup>1 2</sup> Higher concentrations of DFP were needed for the inhibition of true cholinesterase (brain and erythrocyte) than for the pseudocholinesterase (serum). The DFP inhibition was irreversible as opposed to eserine inhibition. With the serum enzyme the presence of DFP did not produce a progressive inactivation, but rather the hydrolysis

DFP concentration, $\times 10^4$	Per cent inhibition*	
	Purified citrus acetylcholinesterase	Crude wheat "lipase"
5	98	91
1	72	75
0.5	56	65

\* After 20 minutes prior exposure of the enzyme to DFP

proceeded as if less enzyme were present from the start and no preliminary incubation of the enzyme with DFP was necessary. Koelle<sup>3</sup> has shown that eserine will protect cholinesterase against DFP inactivation.

The effect of DFP on enzymes of plant origin was studied with the view of determining whether any were more or less specifically inhibited. Of the enzymes jack bean urease, papain, crystalline  $\beta$ -amylase,<sup>4</sup> citrus pectinesterase, and citrus acetylcholinesterase,<sup>5</sup> only the acetylcholinesterase was found to be inhibited by incubation with DFP previous to assay. The characterization of citrus acetylcholinesterase, an enzyme which best hydrolyzes esters of acetic acid and which possesses no true lipase activity, has only recently been reported.<sup>5</sup> The inhibition of this enzyme by DFP is shown in the table.

DFP at  $10^{-3}$  M failed to inhibit the other enzymes under similar conditions. From these results it is apparent that the DFP did not react with

\* Enzyme Research Laboratory Contribution No. 107

<sup>1</sup> Mazur, A., and Bodansky, O., *J. Biol. Chem.*, **163**, 261 (1946)

<sup>2</sup> DuBois, K. P., and Mangun, G. M., *Proc. Soc. Exp. Biol. and Med.*, **64**, 137 (1947)

<sup>3</sup> Koelle, G. B., *J. Pharmacol. and Exp. Therap.*, **88**, 232 (1946)

<sup>4</sup> Balls, A. K., Thompson, R. R., and Walden, M. K., *J. Biol. Chem.*, **163**, 571 (1946)

<sup>5</sup> Jansen, E. F., Jang, R., and MacDonnell, L. R., in press

essential —SH groups, since it failed to inhibit urease and papain, and that the inhibition is specific, since pectinesterase (obtained from the same source as the acetylerase) was not inhibited. The inhibition of wheat "lipase"<sup>6</sup> by DFP is consistent with the previous suggestion that this enzyme is acetylerase.<sup>5</sup>

The reaction of DFP with acetylerase was not dependent upon pH over the range studied (4.9 to 7.5). Dialysis of acetylerase inactivated with DFP failed to restore the activity, hence the reaction is probably irreversible. The kinetics of the reaction show it to be a bimolecular reaction. Longer incubation times required less DFP to produce the same inhibitions reported above.

In the presence of the substrate (0.23 M triacetin) the inhibition reaction proceeded much more slowly for a given concentration of DFP. This decrease in activity with time in the presence of the substrate is opposed to that observed for cholinesterase.<sup>1</sup> Eserine (which has been shown not to inhibit the hydrolysis of acetylcholine by citrus acetylerase<sup>5</sup>) was found not to inhibit the inactivation of acetylerase by DFP.

Similar to its effect on cholinesterase, HETP inhibited acetylerase in lower concentrations than did DFP. With acetylerase only one-fiftieth as much HETP as DFP was needed to cause the same inhibition in a given time. *In situ* inhibition of acetylerase and the resulting effect on metabolism are being investigated.

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Received for publication, July 23, 1947

\* Sullivan, B., and Howe, M. A., *J. Am. Chem. Soc.*, 55, 320 (1933)



# ACTIVITY OF ANTIPERNICIOUS ANEMIA PREPARATIONS IN CHICKS

Sirs

During studies on the factor, present in the soluble components of fish, which stimulates the growth of chicks fed corn-soy bean oil meal diets several liver fractions were tested for activity. Concentrated pernicious anemia preparations were used because of their activity in the dog<sup>1</sup> and we have found that they are highly active in promoting growth of the chick.

Day-old cross-bred chicks from hens on controlled diets were used and fed a basal ration composed of the following: ground yellow corn 35, soy bean oil meal 28, wheat bran 10, wheat middlings 10, casein 7.5, alfalfa

	No. of chicks	Average weight at 4 wks
Basal	12	218
" + 3% fish soluble components	12	307
" + 3% Lederle liver powder, No. DC-1432	12	259
" + 0.1 cc reticulogen (Lilly) 20 U S P units per cc daily*	12	262
Basal	10	209
" + 3% fish soluble components	10	298
" + 0.05 cc reticulogen 20 U S P units per cc daily†	10	301
Basal + 70% methanol extract of fish soluble compo- nents $\cong$ 3%	10	303
Basal + ppt obtained by treating 70% methanol ex- tract with 95% ethanol $\cong$ 3%	10	312
Basal + filtrate from above	10	241

\* Administered orally

† Injected intramuscularly

meal 5, limestone grit 2.0, steamed bone meal 1.5, iodized salt 0.5, fish oil (2000 A, 400 D) 0.5, and  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  0.025 gm, thiamine 0.3, riboflavin 0.6, niacin 5.0, pantothenic acid 2.0, pyridoxine 0.4, inositol 100, choline 150, *p*-aminobenzoic acid 10, biotin 0.02, folic acid 0.05, menadione 0.05, and  $\alpha$ -tocopherol 0.3 mg.

Some of the results are given in the table.

It is evident that highly purified pernicious anemia preparations are as active or more active than the crude liver preparations and that they show

<sup>1</sup> Ruegamer, W. R., Torbet, N., and Elvehjem, C. A., *Federation Proc.*, 6, 287 (1947).

greater activity when injected than when given orally 0.05 cc or 1 U S P unit per day per chick gave a maximum growth response. No differences in hemoglobin levels were found in any of the groups. At present it is impossible to decide whether the active factor is identical with the classical antipernicious anemia factor but the results show that the active factor is soluble in 70 per cent alcohol and precipitated with 95 per cent alcohol. Since the classical pernicious anemia factor follows this fractionation procedure, the chick may be a valuable assay animal for antipernicious anemia preparations.

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Received for publication, July 14, 1947

# THE ACTIVATION OF DEHYDROPEPTIDASE BY ZINC\*

Sirs

In the course of studies on the dehydropeptidase of swine kidney, it was noted that prolonged dialysis of a partially purified enzyme preparation against demineralized water led to an appreciable loss of activity toward the substrate glycyldehydrophenylalanine (GdP). The original activity could be fully restored, however, by the addition of small amounts of zinc chloride.

The enzyme preparation used for prolonged dialysis was obtained by the following procedure. Desiccated and defatted swine kidney powder was extracted with 2 per cent saline and, to the extract, there was added solid ammonium sulfate to 10 per cent saturation. The mixture was filtered and the concentration of ammonium sulfate in the filtrate was raised to 80 per cent saturation. The precipitated proteins were filtered off and dissolved in water. The residual ammonium sulfate was removed by dialysis against distilled water for 2 days at 5°, and the insoluble proteins which separated during dialysis were removed by filtration. The resultant enzyme solution caused a rapid hydrolysis of GdP, as judged by the spectrophotometric assay for dehydropeptidase activity described previously.<sup>1</sup>

The activity of such a solution is markedly reduced following dialysis, for 4 days at 5°, against water which had previously been passed through a Barnstead Bantam demineralizer. In a typical experiment, the proteolytic coefficient  $C_{\text{GdP}}$ <sup>1</sup> was 0.196 before prolonged dialysis and 0.070 after dialysis, the protein content of the solution being unchanged by this treatment. Of the various metals tested, only  $\text{Zn}^{++}$  was found to reactivate the enzyme completely ( $C_{\text{GdP}} = 0.212$ ). At the concentration level used in this study,  $\text{CoCl}_2$ ,  $\text{MnSO}_4$ , and  $\text{MgSO}_4$  were without appreciable effect, the proteolytic coefficients being 0.081, 0.062, and 0.065 respectively. On the other hand, there were noted slight inhibition with  $\text{CdSO}_4$  ( $C_{\text{GdP}} = 0.048$ ) and pronounced inhibition with  $\text{NiSO}_4$  ( $(\text{NH}_4)_2\text{SO}_4$  ( $C_{\text{GdP}} = 0.015$ )).

For the study of the effect of metal ions on dehydropeptidase activity, the enzyme (protein N, 8.1  $\gamma$  per cc) was incubated with the metal salt ( $5 \times 10^{-4}$  M) for 3 hours at 25.6°. The pH of the incubation mixture was 6. To 1 cc of the incubation mixture there were then added 2 cc of 0.1 M veronal buffer (pH 7.9), 1 cc of GdP ( $2.5 \times 10^{-4}$  M), and 1 cc of water. The hydrolysis was conducted at 25.6°, and was followed spectro-

\* This study was aided by grants from the Rockefeller Foundation and the American Cancer Society (on recommendation of the Committee on Growth of the National Research Council).

<sup>1</sup> Yudkin, W. H., and Fruton, J. S., *J. Biol. Chem.*, **169**, 521 (1947).

photometrically as described previously<sup>1</sup>. The comparison blank for the spectrophotometric readings contained the same components as did the test system, except that the substrate solution was replaced by water.

Previous studies<sup>1</sup> have shown that dehydropeptidase is completely inhibited by  $6 \times 10^{-4}$  M sulfide, and only partially inhibited by  $10^{-3}$  M cyanide or cysteine. These results suggested the possibility that dehydropeptidase belongs to the group of metal-containing enzymes. Berger and Johnson<sup>2</sup> and Smith and Bergmann<sup>3</sup> have provided evidence for the metal-protein nature of several peptidases and, in particular, Smith<sup>4</sup> has studied the activation of L-leucine aminopeptidase by manganese ions. The results presented in the present communication support the view that zinc ions may be involved in dehydropeptidase action. It is premature to conclude that the dehydropeptidase of swine kidney is a zinc-protein, since activation by a metal ion does not necessarily imply that the metal constitutes a part of the active enzyme.<sup>5</sup> This question may be clarified by further studies, which are in progress.

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Received for publication, August 12, 1947

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## BIOTIN ACTIVATION OF CERTAIN DEAMINASES

Sirs

In a previous paper<sup>1</sup> we have demonstrated that biotin is involved in the decarboxylation of oxalacetate. It has now been found that the deaminases for aspartic acid, serine, and threonine may be resolved by the same techniques used previously (exposure of the living bacterial cells to m phosphate at pH 4 for 30 minutes at 27-37°), and that the lost activity is restored specifically by biotin. The data in the table indicate that a mixture of vitamins lacking in biotin has little or no effect, while the same mixture with added biotin effectively activates the resolved enzymes. Biotin alone is virtually as active as the vitamin mixture. The reversal of the aspartic deaminase shows a similar response to biotin. These results demonstrate a second rôle for biotin which may help to explain the results of previous investigators.<sup>2</sup> (See following page for the table.)

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Received for publication, July 26, 1947

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*Biotin Activation of Deaminases*

Cells of organisms grown 16 to 20 hours at 27° in media containing 1 per cent each of yeast extract and peptone, and 0.5 per cent phosphate, except cells of the Gratia strain of *E. coli* which were grown with addition of 0.3 per cent glucose and 0.1 per cent formate. Cells harvested by centrifugation, washed with distilled water, and suspended in M phosphate, pH 4, for 30 minutes at 27°. Reaction run at 37° (volume 2 ml) in 0.5 M phosphate at the pH indicated. Amino acids added at 0.005 M final concentration. Reaction stopped at 20 or 30 minutes with trichloroacetic acid, centrifuged, and an aliquot of the supernatant analyzed for ammonia by nesslerization in a photoelectric colorimeter. The increase in ammonia over samples stopped at 0 time was taken as an index of deamination.

Organism		Deamination pH	CNH <sub>3</sub> (N)			
			No additions	Vitamins less biotin*	Vitamins†	Biotin, 0.1 γ per ml
<i>E. coli</i> (Gratia)	L-Aspartic acid	4	96	108	334	304
		4	73	92	260	239
		4	27	29	116	102
		4	131	132	286	286
		5	220	241	583	571
		7	419	467	735	752
<i>P. vulgaris</i>		7	28			277
		7	100	121		377
<i>E. coli</i> (86G)		7	58			114
		7	154	154		279
<i>B. cadaveris</i>		7	49			120
		7	76	84		238
<i>E. coli</i> (Gratia)	L-Serine	7	89			159
		7	69			117
<i>P. vulgaris</i>		7	99			194
		7	273	270		488
<i>E. coli</i> (86G)		7	124			172
		7	258	260		403
<i>B. cadaveris</i>		7	39			95
		7	117	117		249
<i>E. coli</i> (86G)	DL-Threonine	7	52			122
		7	178	175		290
<i>B. cadaveris</i>		7	29			78
		7	45	56		161

\* Vitamins added per ml: nicotinic acid 2.5 γ, *p*-aminobenzoic acid 1 γ, riboflavin 0.5 γ, pantothenic acid 0.5 γ, thiamine 1 γ, folic acid 0.5 γ, pyridoxal 5 γ.

† As vitamins less biotin with free biotin added to yield 0.1 γ per ml of reaction mixture. We are indebted to Merck and Company, Inc., for some of the vitamins and to the Lederle Laboratories for the synthetic folic acid.

# THE RESOLUTION AND PURIFICATION OF GLUTAMIC-ASPARTIC TRANSAMINASE

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(Received for publication, June 7, 1947)

In recent studies on transamination Green, Lelou, and Noeito (1) have clearly shown that the glutamic-aspartic and glutamic-alanine systems involve two separate enzymes, and have prepared both in a highly purified state. Schlenk and Fisher (2) have purified the glutamic-aspartic transaminase by an independent procedure. After Snell (3) had postulated the transaminase function of pyridoxal and pyridoxamine from chemical analogies, and Schlenk and Snell (4) had presented excellent presumptive data for the function of vitamin B<sub>6</sub> in transamination, Lichstein, Gunsalus, and Umbreit (5) were able to demonstrate the function of pyridoxal phosphate as the coenzyme of the glutamic-aspartic transaminase of bacterial cells. Green *et al.* (1) were able to demonstrate codecarboxylase in the purified transaminases by means of the dopa carboxylase enzyme. In the bacterial studies (5) the apotransaminase was obtained both by resolution of the holoenzyme and by the growth technique in which apoenzyme is obtained by harvesting cells from a vitamin-deficient medium (6). Kritzman and Samarina (7) have shown that pyridoxal phosphate will reactivate the partially resolved glutamic-alanine transaminase from pig heart. In preliminary studies employing the apoenzyme of deficient bacterial cells, data were obtained (8) which agreed with Snell's postulate of the mechanism of coenzyme action.

In this study the glutamic-aspartic transaminase from pig heart has been completely resolved and the apoenzyme obtained in a high state of purity. Pyridoxal phosphate acts as the coenzyme of the resolved transaminase.

## Methods

Green *et al.* (1) have devised improved methods for the study of transamination which afford more rapid assay for the enzyme activity. Whenever possible, their methods were used. A few alterations were necessary for the assay of the apoenzyme instead of the holoenzyme studied by these workers.

**Enzyme Assay**—The formation of oxalacetic acid by the glutamic-aspartic transaminase was measured as follows

The main compartment of the Warburg cup contained 0.5 ml of 0.2 M phosphate buffer, pH 7.3, enzyme, coenzyme, usually 10  $\gamma$  of pyridoxal phosphate, and water to a volume of 1.7 ml. These were incubated for 10 minutes at 37° and the neutralized substrates added from the side arm, in this case 0.25 ml each of 0.8 M L-aspartic acid and 0.4 M  $\alpha$ -ketoglutaric acid. After a 10 minute reaction period, 0.5 ml of aniline-citrate was added from the second side arm, and the gas evolution (from oxalacetic acid) read after 10 minutes. This procedure differs from the method of Green *et al* (1) in the addition of both substrates from the side arm after an equilibration period. As shown below, this change was necessitated by the time required for the enzyme-coenzyme complex to reach maximum activity. It should be borne in mind that the assays reported in this paper are for the total enzyme, the holoenzyme content may be calculated from the per cent resolution.

Because ammonium sulfate inhibits transaminase activity (1), all samples were dialyzed before assay.

**Coenzyme**—Synthetic barium pyridoxal phosphate (9) was used throughout these studies. In some cases the barium was removed with an equivalent amount of sulfate ion and the coenzyme neutralized to pH 7 and preserved by drying aliquots in a lyophile apparatus.

**Enzyme Units and Purity Index**—The methods of Green *et al* of expressing the enzyme units and the purity index were used. 1 unit of glutamic-aspartic transaminase is that amount of enzyme which will form oxalacetic acid equivalent to 100 microliters of carbon dioxide in 10 minutes at 37° (Green's experiments were performed at 38°).

The purity index is based on the light extinction at 280 m $\mu$  in a 1 cm cell, divided by the enzyme units.

**Enzyme Preparation**—An outline of the method of purification and resolution of the glutamic-aspartic transaminase is shown in the accompanying diagram. 6 kilos of frozen pig heart were ground four times through a fine mesh food chopper and extracted at room temperature with 4 times its weight of distilled water for 1 hour. The solids were removed by straining through cheese-cloth, were reextracted with 100 ml of distilled water per 100 gm, and again removed. The combined supernatants were filtered through coarse paper and refiltered through Schleicher and Schull No. 595 paper until clear. 30 gm of solid ammonium sulfate per 100 ml were added with stirring at room temperature. The precipitate was allowed to settle in the refrigerator overnight and collected by filtration. The filtrate containing the glutamic-aspartic enzyme was treated with an additional 40 gm of ammonium sulfate and, after settling, the precipitate was collected by filtration. This precipitate was suspended along with the filter paper in 3 liters of distilled water at room temperature and stirred mechanically until the filter paper was disintegrated and the



*Flow Sheet for Purification of Glutamic Aspartic Transaminase*Ground pig heart, extract with 100 ml H<sub>2</sub>O per 100 gm, filterExtract with 100 ml H<sub>2</sub>O per 100 gm, filter

Discard

+ 30 gm (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> per 100 ml (39% saturation)Suspend in H<sub>2</sub>O,  
filter+ 10 gm (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> per 100 ml (92% saturation)

Discard

Glutamic alanine  
enzymeSuspend in H<sub>2</sub>O, filter

Discard

Discard

Add (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 46 gm per 100 ml (60% saturation)Suspend in H<sub>2</sub>O, filter

Discard

Discard

Heat to 60°, cool,  
filter

Discard

+ 4 volumes Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> gel, 30 min,  
centrifuge

Discard

+ 70 gm (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (92% saturation),  
filterSuspend in H<sub>2</sub>O, filter

Discard

Discard

Dialyze, filter

Discard

Glutamic-aspartic transaminase  
(98% resolved, purity index 0.016)

precipitate dissolved. The resulting suspension was filtered with suction and the enzyme reprecipitated by the addition of ammonium sulfate to a concentration of 46 gm per 100 ml (assuming the precipitate to be saturated with ammonium sulfate). The precipitate was removed by filtration and the precipitate, plus the filter paper, was suspended in 250 ml of distilled water, the paper was broken up by mechanical stirring and removed by filtration with suction. The precipitate was washed with 100 ml of distilled water, filtered, and the two filtrates combined. The combined filtrates were heated to 60° in a boiling water bath and immediately cooled in ice water and filtered to remove the coagulum. After dialysis for 12 hours, further purification was accomplished by adsorption of impurities with tricalcium phosphate gel, the amount required to

TABLE I  
*Purification and Resolution of Glutamic-Aspartic Transaminase*

Preparation No	Steps in preparation	Enzyme units per 100 gm pig heart	Purity index	Resolution
				per cent
1	H <sub>2</sub> O extract	22,900	0.18	0
	Supernatant, 30 gm (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	4,800	0.31	57
	Ppt, 70 gm (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	4,500	0.16	73
	Reprecipitate, 46 gm (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5,000	0.09	99
	Heated to 60°, filtered, dialyzed	3,000	0.05	98
	Supernatant, Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> gel	970	0.037	96
	Ppt, 70 gm (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	940	0.016	98
2	H <sub>2</sub> O extract	27,700	0.14	17
	Supernatant, 30 gm (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	19,500	0.08	59
	Ppt, 70 gm (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	12,200	0.06	68
	Reprecipitate, 46 gm (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	13,400	0.06	97

adsorb the impurities being determined in a pilot experiment. After the gel was added, the mixture was stirred for 30 minutes, centrifuged, and the supernatant decanted. The enzyme was precipitated from the supernatant solution with 70 gm of ammonium sulfate per 100 ml. After flocculation, the precipitate was recovered by centrifugation, dissolved in water, and dialyzed 24 hours against distilled water. The small amount of precipitate which formed upon dialysis was removed by centrifugation. In this stage of purification the apoenzyme was more highly purified than the holoenzyme samples so far prepared (1, 2).

The enzyme units recovered at each stage of purification, the percentage resolution of the enzyme, and its purity index are indicated in Table I.

A second sample of enzyme was carried through enough steps of the purification to confirm the resolution (Table I). With this sample, how-

ever, the ammonium sulfate was removed from the first glutamic-aspartic precipitate by dialysis before reprecipitation with the 46 gm. of ammonium sulfate. Only about one-third of the enzyme was lost with the first 30 gm. of ammonium sulfate precipitate, as compared to nearly three-fourths lost in the first experiment. Also, it should be noted that in the second preparation the yield of enzyme at each stage and the purity index were higher than in the first experiment. With these increases, one would predict that in the neighborhood of 10 per cent of the enzyme present in the first aqueous extract could have been obtained in the purified state, as compared to about 1 per cent in the first preparation.

Several differences in enzyme recovery, resolution, and the purity index exist between the data reported in this paper and those of Green *et al* (1) and of Schlenk and Fisher (2).

The transaminase activity obtained in the first water extracts were from 23,000 to 27,000 units per 100 gm. of pig heart in the experiments reported here, as compared to 6000 in Green's and about 1600 in the Schlenk and Fisher preparation.

Inasmuch as Green lost 85 per cent of the enzyme in the first precipitation step and Schlenk and Fisher recovered only 5 per cent of the enzyme present in the acetone-dried pig heart, it seems possible that they also encountered resolution. This is suggested especially by the 70 per cent resolution of the enzyme by the time of the first precipitation (Table I). As pyridoxal phosphate was not available at the time their experiments were started, it would not be surprising if this went unnoticed.

The purity of the preparations also differed. Green's best preparation had a purity index of 0.037, Schlenk's 0.02, and the apoenzyme reported here 0.016. Since a higher state of purity can be obtained with apoenzyme than has been possible so far with the holoenzyme, it seems possible that the latter either contains some apoenzyme or is contaminated with non-specific protein. Very probably the apoenzyme has not been obtained in a pure state, especially since not all of the steps which were found beneficial for the preparation of the holoenzyme have been used.

To add still a third hypothesis for the presence of fractions with different electrophoretic mobilities in the purified transaminase of Green *et al*, it is suggested that the inactive fraction corresponds to the apoenzyme.

#### *Activity of Glutamic-Aspartic Apotransaminase*

During the resolution of the glutamic-aspartic enzyme the reproducibility of the activity determinations was not as close as desirable. A study of the cause of this difficulty showed that it could be eliminated by incubating the coenzyme with the enzyme before the substrates were added. This appeared to be due to the time required for coenzyme-

enzyme complex formation As shown in Fig 1, the presence of aspartic acid, as in the usual assay, retarded the rate at which this equilibrium was attained If the  $\alpha$ -ketoglutaric acid was added first and the aspartic acid tipped from the side arm, the enzyme activity as estimated was still less As the curve shows, the equilibrium was approximately complete in 10 minutes if 10  $\gamma$  of coenzyme were incubated with the enzyme, and was not complete even at the end of 40 minutes if either of the substrates was present during the incubation period On the other hand, if the coenzyme concentration was increased to 100  $\gamma$  per cup, the equilibrium was established within 10 minutes, regardless of the order of addition of

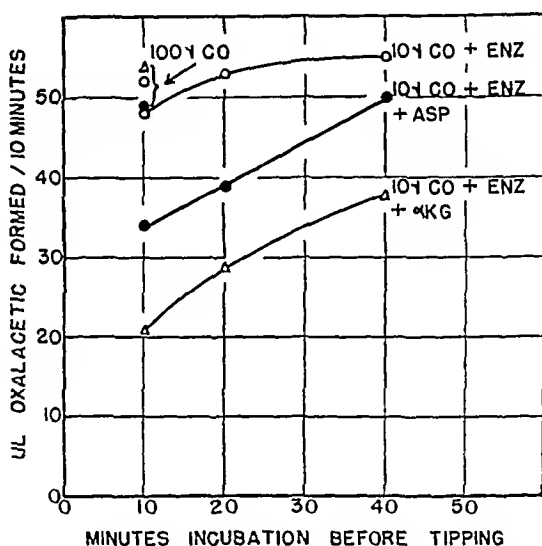


FIG 1 Influence of substrates upon the rate of enzyme coenzyme complex formation Concentrations as indicated in "Methods" Pyridoxal phosphate, enzyme, and substrate incubated as indicated, omitted substrates tipped from the side arm

the substrates Increasing the substrate concentrations resulted in low assay values, especially with 10  $\gamma$  of coenzyme

In order to conserve coenzyme and because satisfactory assays could be obtained, the 10 minute incubation period was used with 10  $\gamma$  of coenzyme

The coenzyme saturation curve for the glutamic-aspartic apotransaminase is shown in Fig 2 The dissociation constant (Michaelis) calculated by the Lineweaver-Bulk method (10) is  $1.5 \times 10^{-6}$  The coenzyme concentration at half maximum activity is 0.75  $\gamma$  of pyridoxal phosphate per ml The dissociation constant is comparable to the  $K = 2.1 \times 10^{-6}$  found for the purified tryptophanase enzyme of *Escherichia coli* (11)

The dissociation constant of pyridoxal phosphate for the tyrosine decarboxylase<sup>1</sup> is  $1.5 \times 10^{-3}$ . Whether this difference is due to a difference in the nature of the enzymes or whether the pH affects the dissociation was not determined. The tyrosine decarboxylase reaction is run at pH 5.5, the transaminase at 7.5, and the tryptophanase at 8.3.

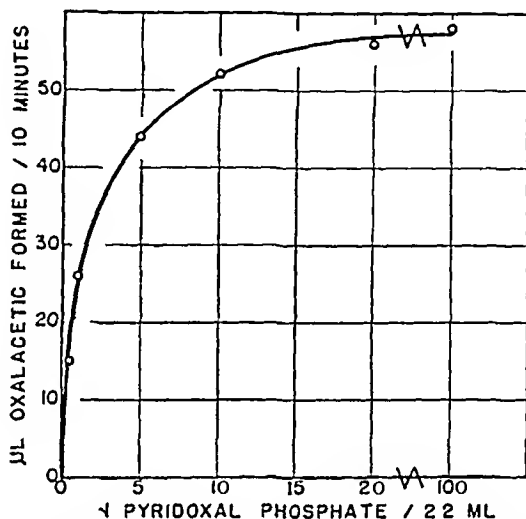


FIG 2 Pyridoxal phosphate saturation curve of glutamic-aspartic transaminase

#### SUMMARY

The transaminase enzyme for the amino acid pair glutamic-aspartic, and their respective keto acids,  $\alpha$ -ketoglutaric-oxalacetic, has been resolved.

In the process of resolution the apoenzyme has been obtained in a higher state of purity than that previously attained with the holoenzyme.

Pyridoxal phosphate serves as coenzyme for this enzyme, the dissociation constant being  $1.5 \times 10^{-6}$  moles per liter.

The data presented confirm the function of pyridoxal phosphate in transamination, as demonstrated with the apoenzyme from bacterial cells and by indirect methods of analysis of purified holoenzyme from pig heart.

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# ASPARTIC-ALANINE TRANSAMINASE, AN ARTIFACT

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(Received for publication, June 7, 1947)

Reports on transamination by Braunstein (1) and coworkers have dealt mainly with two reactions, referred to as the aspartic system and the glutamic system, each of which transfers an amino group to pyruvic acid with the formation of alanine. Transamination studies in this country, largely by Cohen (2), gave little evidence for the occurrence of the aspartic system, but the glutamic system (3) was studied and an additional enzyme, which catalyzes the interconversion of glutamic and aspartic acids with their respective keto acids, was reported. More recently, Green and coworkers (4) have separated and purified the glutamic-alanine and the glutamic-aspartic enzymes, and have thus demonstrated that two distinct enzymes are involved. In connection with studies of the vitamin B<sub>6</sub> group (5), pyridoxal phosphate has been shown to function as the coenzyme of both the glutamic-aspartic (6) and the glutamic-alanine (4, 7) enzymes.

Kritzmann (8) has partially purified the aspartic system, in Green's nomenclature aspartic-alanine system, and has found that dialysis, precipitation, or adsorption of the enzyme results in loss of activity. The activity was partially restored by a concentrate of boiled muscle juice (8) and the active agent partially purified (9).

In view of the lack of evidence for an aspartic-alanine transaminase in the work of the American investigators, and as a result of the recent purification of the glutamic-aspartic and glutamic-alanine enzymes, Green *et al.* (4) have suggested that the aspartic-alanine activity may occur through the combined action of these two enzymes, with either glutamic acid or  $\alpha$ -ketoglutaric acid acting as a link. Glutamic acid would answer the description of Braunstein and Kritzmann's coenzyme (9).

In the attempts to demonstrate the aspartic-alanine reaction it was found that the activity of this system in aqueous extracts of ground pig heart is slight as compared to the activity of the glutamic-aspartic and glutamic-alanine systems. The aspartic-alanine activity can be reproduced by a mixture of the purified glutamic-alanine and glutamic-aspartic enzymes in the presence of pyridoxal phosphate and glutamic acid, or of pyridoxal phosphate and heart concentrate.

## Methods

*Activity of Transaminases*—Kritzmann (8) has followed the activity of the aspartic-alanine transaminase by measuring the formation of the

monocarboxylic amino acid in the presence of aspartic and pyruvic acids as substrates. Because of greater convenience we have measured the oxalacetic acid formed manometrically (4). The aspartic-alanine activity was measured as follows. The main compartment of the Warburg flask contained 0.5 ml of 0.2 M phosphate buffer, pH 7.3, 100  $\gamma$  of barium pyridoxal phosphate, enzyme, and 0.2 ml of heart concentrate or 1.2 mg of glutamic acid in a total volume of 2.0 ml; these were incubated for 10 minutes, and 0.25 ml of 0.8 M L-aspartic acid and 0.25 ml of 0.4 M pyruvic acid, both adjusted to pH 7.3, were added from the side arm. The reaction was allowed to proceed for 90 minutes at 37° and at the end of this period 0.5 ml of aniline-citrate was added from the second side arm. After a 10 minute period the carbon dioxide, released from the oxalacetic acid, was measured.

The glutamic-aspartic activity of the enzyme preparations was measured by the method of Green *et al* (4), as modified by O'Kane and Gun-salus (10).

The concentration of the glutamic-alanine enzyme was determined by following the formation of pyruvic acid with the salicylaldehyde method, as used by Green *et al* (4).

*Coenzymes*—Barium pyridoxal phosphate was used to reactivate the system, especially after the first precipitation steps in which the glutamic-aspartic system had become resolved (10).

Braunstein and Kritzmann's coenzyme (9), boiled muscle juice concentrate, was prepared as follows. 300 gm of finely ground defatted pig heart were mixed with an equal weight of water in a Waring blender for 1 hour at room temperature. This finely ground tissue was heated in a boiling water bath until the temperature of the suspension reached 90° and was held for 5 minutes. The coagulum was removed by filtration with a Buchner funnel to yield 350 ml of filtrate. The filtrate was chilled, the fat removed, and the clear supernatant concentrated *in vacuo* at around 50° to a volume of 60 ml. 0.2 ml of this concentrate was equivalent to 1.0 gm of ground tissue. An assay of this preparation for pyridoxal phosphate, with the tyrosine decarboxylase apoenzyme (11), showed 44 m $\gamma$  of barium pyridoxal phosphate activity per 0.2 ml (per gm of tissue).

The boiled heart concentrate was also analyzed for glutamic acid, with an acetone-dried preparation of *Escherichia coli* (12) which contained only glutamic acid decarboxylase. 0.2 ml of the heart concentrate contained 180 microliters (1.2 mg) of glutamic acid.

*Extraction of Transaminases by Method of Kritzmann*—830 gm of defatted frozen pig heart were ground through a fine mesh food chopper five times. The minced muscle was suspended in 830 ml of distilled



water and allowed to stand in the refrigerator for 3 hours with occasional stirring. The suspension was then divided into two portions. One portion was centrifuged to remove the solids and the supernatant was decanted. Both preparations were autolyzed at 37° and the aspartic-alanine activity determined with and without the addition of heart concentrate.

This method of preparation yields much more concentrated extracts than do those employed by other workers. Cohen's (3) method for preparing the transaminases involves several washings of the ground tissue, followed by extraction of the enzymes with 5 volumes of 1 per cent potassium bicarbonate, the method of Green *et al.* (4) employs 4 volumes of water for extraction.

TABLE I

*Comparative Transaminase Activity of Water Extract of Ground Heart Muscle*

Conditions as described for each system in "Methods," enzyme, the supernatant from extraction of ground tissue with equal weight of water

Substrate	Enzyme used ml	Enzyme, units per ml	Enzyme units per 100 gm tissue
Aspartate, $\alpha$ ketoglutarate	0.01	107	52
+ 10 $\gamma$ pyridoxal phosphate		200	97
Aspartate, pyruvate*	0.5	0.16	0.22
+ 10 $\gamma$ pyridoxal phosphate		0.66	0.32
+ Heart concentrate		0.58	0.28
+ 10 $\gamma$ pyridoxal phosphate and heart concentrate		1.32	0.64
Glutamate, pyruvate	0.01	19	9.3

\* Units expressed as 100 microliters of CO per 10 minutes, assay run 90 minutes

#### EXPERIMENTAL

The activity of the supernatant from the aqueous extract of ground pig heart muscle for the three transaminase systems is compared in Table I. It should be noted that 0.01 ml of the aqueous extract is sufficient for the assay of glutamic-aspartic and glutamic-alanine enzymes, whereas 0.5 ml is required for the aspartic-alanine assay. In addition, a 10 minute incubation period is sufficient for the former, whereas a 90 minute period is required with the latter to detect activity. As indicated in Table I, the aspartic-alanine activity is only about 0.5 per cent that of the glutamic-aspartic and 2 per cent of the glutamic-alanine activity.

Kutzmarn (8) has suggested that the aspartic-alanine enzyme activity may be increased by autolyzing the ground tissue suspension for several days at 0°, or for several hours at 37°. The results of such treatment and

the influence of the Braunstein and Kritzmann heart concentrate are shown in Table II. Autolysis of the ground pulp for 27 hours at 37° resulted in almost twice the activity of the freshly ground tissue, the autolysis largely replaced the effect of the boiled tissue concentrate. Incubation of the extract, obtained after removing the pulp, did not result in increased activity (Table II). Dialysis reduced the activity by about one-half, full activity being restored by the addition of the heart concentrate.

Separation of the enzymes was accomplished by ammonium sulfate precipitation. The tissue suspensions were autolyzed, the pulp removed, and the filtrate heated to 56° to precipitate meat proteins. The transaminase activity of the three systems is shown in Table III. It may be seen that better than 50:1 separation of the glutamic-aspartic from the

TABLE 11

*Influence of Autolysis at 37° on Aspartic-Alanine Transaminase Activity*  
Conditions as described under "Methods," enzyme, 0.5 ml. of the tissue filtrate

Autolysis at 37	Oxalacetate formed per 90 min			
	0 hr	7 hrs	19 hrs	27 hrs
	microliters	microliters	microliters	microliters
Tissue brei	230	313	398	422
" " + heart concentrate	364	395	447	451
Extract (pulp removed)	232	172	170	175
" + heart concentrate	331	323	317	317
" after dialysis	100			
" + heart concentrate	300			

glutamic-alanine systems was obtained by the third precipitation, where traces of aspartic-alanine activity existed in both fractions. Heart concentrate always increased the aspartic-alanine activity, and pyridoxal phosphate was necessary for the glutamic-aspartic enzyme. The glutamic-alanine enzyme was not resolved, i.e., addition of pyridoxal phosphate or heart concentrate did not influence its activity. After the first precipitation both heart concentrate and pyridoxal phosphate were required for maximum aspartic-alanine activity.

In further attempts to purify the aspartic-alanine system and to separate it from the two enzymes described by Green, it was found that traces of activity followed both fractions, with a larger amount occurring in the glutamic-aspartic fraction. Since the purified glutamic-alanine and glutamic-aspartic enzymes had been prepared for other experiments, this fractionation was not carried further.

TABLE III  
Fractionation of Transaminase Activity

Fraction	Total units				
	Glutamic aspartic		Aspartic alanine		Glutamic alanine
	No additions	10 $\gamma$ pyridoxal phosphate	No additions	10 $\gamma$ pyridoxal phosphate + heart concentrate	No additions
Water extract, as in Table I	17,700	33,000	85	226	3100
1st fractionation, Ppt 30 gm $(\text{NH}_4)_2\text{SO}_4$	585	2,665	11	52	2850
1st " " 70 " "	4,180	16,330	9	28	295
2nd " " 30 " "	120	380	4	23	1430
2nd " " 70 " "	800	10,000	10	23	None
3rd " " 2 $\times$ 25 gm $(\text{NH}_4)_2\text{SO}_4$	None	None	0.4	0.8	225
3rd " " 35 gm $(\text{NH}_4)_2\text{SO}_4$	600	3,652	3	5	None
25 gm + 35 gm $(\text{NH}_4)_2\text{SO}_4$ ppts			7.4	23.4	

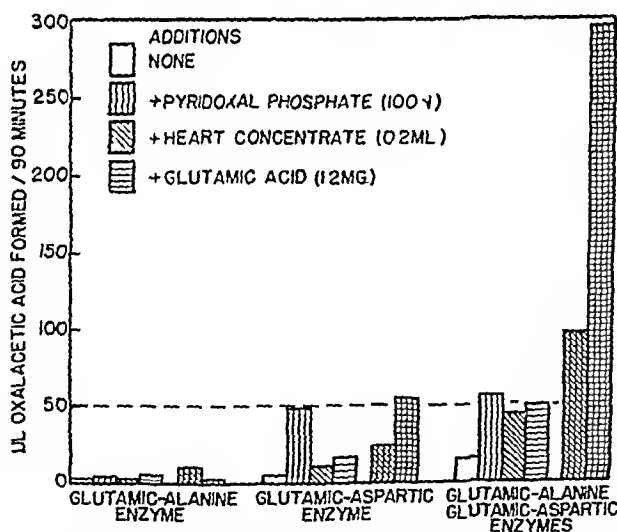


FIG 1 Aspartic alanine activity of purified transaminases. 0.3 ml of glutamic-alanine (12 units per ml) and of glutamic aspartic (22 units per ml) were used. Concentrations as described under "Methods."

The results of testing the purified systems for aspartic-alanine activity, singly and in combination, are shown in Fig 1. The purified glutamic-aspartic system in a concentration of 0.3 ml per Warburg cup was prac-



# LIPOLIDASE ACTIVITY OF PORK HEMOGLOBIN AND MUSCLE EXTRACT\*

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(Received for publication, June 9, 1947)

The development of rancidity and the disappearance of the pink color of stored pork are serious problems in the meat industry. The rancid meat has an unpleasant odor and flavor. Also the rancid fat is mildly toxic and destroys food accessories such as vitamin A, niotene, vitamin E, vitamin D, pantothenic acid, pyridoxine, and biotin (1). The development of rancidity is due to the oxidation of unsaturated fatty acids in the pork (2). The disappearance of the pink color is due to the decomposition of hemoglobin (3). The two reactions are correlated. Rancidification is accompanied by the transformation of the original pink color of pork to gray or brownish gray (4).

Lea (5) demonstrated the presence in pork muscle of a lipoxidase capable of accelerating peroxidation of unsaturated fatty acids of pork fat. The enzyme was heat-labile and its activity was influenced by the pH of the medium, falling off from high values below pH 5.3 to zero activity at pH 7. No further work has been reported on identification of this enzyme.

The lipoxidase activity of iron porphyrins has been established independently by a number of workers (6-10). Robinson (6) demonstrated a marked acceleration of oxygen uptake by linseed oil in the presence of hemoglobin, methemoglobin, and hemin, but no acceleration by iron-free hematoporphyrin. Haurowitz and coworkers (7) showed that peroxidation of unsaturated fatty acids by hemins was accompanied by destruction of the hemin molecule with loss of color. Since pork contains both hemoglobin and the closely related myoglobin, it might be assumed that the lipoxidase activity of pork muscle juice could be ascribed to its content of these hemin compounds. Rancidification and accompanying discoloration of raw pork (4) could be explained on the same basis.

However, little positive evidence exists for the identity of hemoglobin (or myoglobin) and pork lipoxidase. There are no comparisons of the

\* Published as Scientific Paper No. 724, College of Agriculture and Agricultural Experiment Stations, Institute of Agricultural Sciences, State College of Washington, Pullman, Washington. Taken from a thesis presented by Da-Hwei Peng as partial fulfillment of the requirements for the degree of Master of Science, State College of Washington, 1947.

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A portion of filtrate containing approximately 0.5 gm of fat was then used for the determination of the peroxide number by a slight modification of the Wheeler method (14). The peroxide number was expressed as mm of peroxide per 1000 gm of fat.

*Phosphate Buffer Blank*—Peroxide numbers were also run on a series of phosphate buffer-lard mixtures at various pH values in order to correct values obtained with muscle extract or hemoglobin. The phosphate buffer blanks were made by mixing 10 gm of the carotene-lard mixture and 1.5 ml of diluted phosphate buffer solution (1 part of 0.2 M phosphate buffer and 2 parts of distilled water). The mixture was treated in the same manner as the lard with added muscle extract or hemoglobin solution.

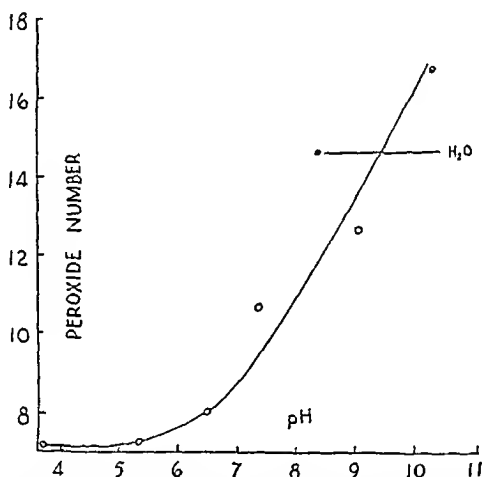


FIG 1 Peroxide number of lard-phosphate buffer mixture after 72 hours. The horizontal line indicates the peroxide number of the distilled water-lard mixture.

### Results

*Effect of Phosphate Buffers on Oxidation of Lard*—Phosphate inhibited lard oxidation at low pH values, but the inhibitory effect was rapidly lost at pH values above 6.5 (Fig. 1). Similar results were reported by Lea (15).

*Catalytic Effect of Muscle Extract and Hemoglobin on Oxidation of Lard at Various pH Values*—Preliminary experiments on muscle extract and hemoglobin solutions carried out at the original pH of these solutions (approximately 5.8 for muscle extract and 7.0 for hemoglobin) without added buffer showed a marked catalytic effect on the oxidation of lard in both cases. The hemoglobin caused more rapid oxidation than the muscle extract in all cases, but, since the concentration of hemoglobin was not ad-

justed to that of the muscle extract, part at least of the observed differences were undoubtedly due to concentration differences.

The addition of phosphate buffer (pH 5.8) to a muscle extract at the same original pH reduced the rate of oxidation over that obtained from the same concentration of muscle extract alone. Approximately the same inhibition was obtained with lard-phosphate systems at this pH (see Fig. 1).

Fig. 2 shows the effect of pH on the lipoxidase activity of muscle extract in phosphate buffers. The peroxide numbers of the buffer-lard control

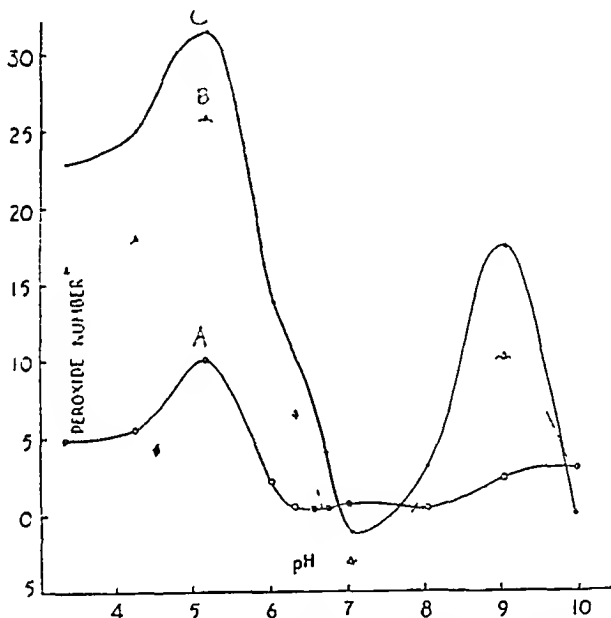


FIG. 2 Effect of pH on oxidation of muscle extract lard combinations at various time intervals. Curve A, 68 hours, Curve C, 141 hours, Curve B, the average of results at four time intervals.

at each pH were subtracted from the muscle extract values before plotting. The "hemoglobin" content of the muscle extract was 0.137 gm per 100 ml. The peroxide numbers were determined at several intervals (68, 114, 141, and 164 hours). The average values of the four determinations of peroxide number were calculated and plotted as a function of pH.

The catalytic effect of muscle extract was greatly influenced by the pH. The accelerating effect of acid in the pH range of 5 to 7 was similar to that reported by Lea (5). Lea did not extend his observations beyond this range. On the acid side of the curve, the maximum activity of muscle ex-

*Heat Inactivation of Hemoglobin and Muscle Extract*—This experiment compared the effect of heat on the lipoxidase activity of muscle extract with its effect on hemoglobin at the same pH (5.3). 10 ml of phosphate buffer solution and an equal quantity of muscle extract or hemoglobin solution were pipetted into a small beaker. The mixture was heated rapidly on a boiling water bath to the desired temperature. After cooling, the precipitate was ground in a mortar and 1.5 ml of the suspension were added to 10 gm of carotene-lard. The results are shown in Figs 4 and 5.

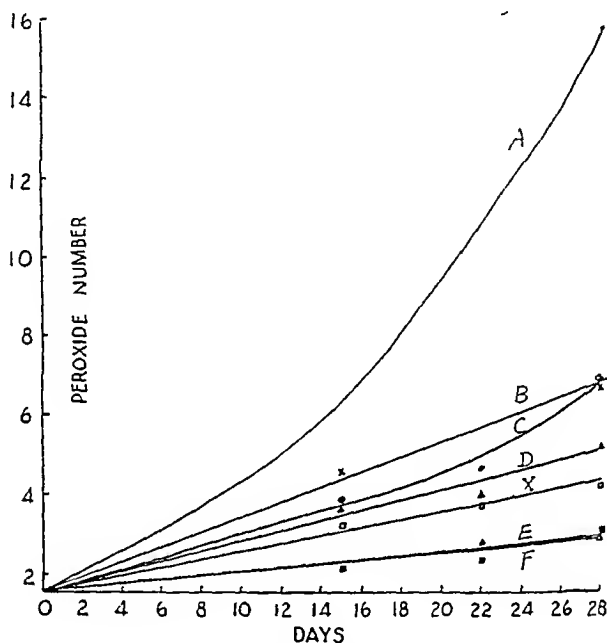


FIG 4 Effect of heating muscle extract at pH 5.3 on its lipoxidase activity. Curve A, unheated, Curve B, 60° for 30 minutes, Curve C, 70°, Curve D, 80°, Curve E, 90°, Curve F, 70° for 15 minutes, and Curve X, distilled water control.

The effect of heat on the muscle extract was similar to that on hemoglobin. Heated to 70°, only a part of the catalytic effect of muscle extract or hemoglobin was destroyed, but at 90° a flocculent coagulum appeared and no catalytic activity remained. When muscle extract or hemoglobin was held at 70° for 15 minutes, precipitation occurred and their catalytic effects were destroyed completely.

Rancidity development in frozen cooked pork sausage has been shown to be independent of the pH. Cooked sausage turned rancid much more slowly than raw sausage, except at the highest pH of normal pork (4).

*Effect of Dilution on Lipoxidase Activity of Muscle*—Fig 6 shows the effect of diluting muscle extract and hemoglobin at the same pH (5.3).



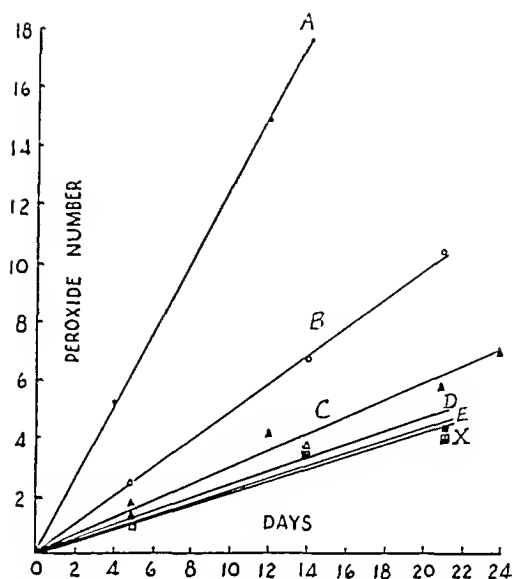


FIG 5 Effect of heating hemoglobin solution at pH 5.3 on its lipoxidase activity Curve A, unheated, Curve B, 70°, Curve C, 80°, Curve D, 90°, Curve E, 70° for 15 minutes, and Curve X, distilled water control

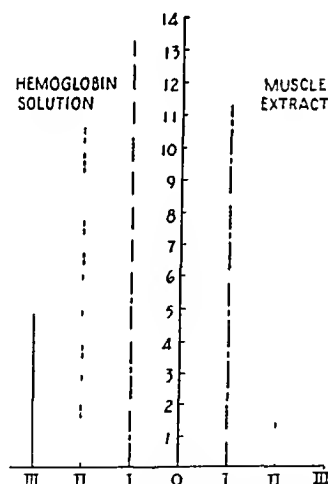


FIG 6 Effect of dilution on the lipoxidase activity of muscle extract and hemoglobin after 115 hours Curves I, I', no dilution, Curves II, II', one-third dilution, Curves III, III', two-thirds dilution

A 10 gm portion of carotene-lard was mixed with 15 ml of solution. The solutions were prepared in three different dilutions. In Curve I,

the solution contained 3 ml of phosphate buffer solution and 3 ml of muscle extract or hemoglobin, in Curve II, 3 ml of phosphate buffer solution, 1 ml of distilled water, and 2 ml of muscle extract or hemoglobin solution, in Curve III, 3 ml of phosphate buffer solution, 2 ml of distilled water, and 1 ml of muscle extract or hemoglobin solution

The concentrations of muscle "hemoglobin" and blood hemoglobin were the same, 0.103 gm per 100 ml. The activities of both hemoglobin solution and muscle extract decreased with decreasing concentrations. As previously shown (Fig 3), hemoglobin caused only a slightly greater increase in oxidation of the fat than that brought about by undiluted muscle extract at this pH. However, the effect of muscle extract fell off more rapidly than that of the hemoglobin solution on dilution.

TABLE I

*Effect of Cyanide on Lipoxidase Activity of Muscle Extract and Hemoglobin Solution Exposed in Darkness*

Solution added to lard	Peroxide No., 14 days
Muscle extract	3.8
" " + KCN	4.4
Hemoglobin solution	3.3
" " + KCN	3.2

TABLE II

*Effect of Cyanide on Lipoxidase Activity of Hemoglobin Solution Exposed in Room Light*

Solution added to lard	Peroxide No., 3 days
Hemoglobin solution	22.7
" " + KCN	21.4

It seems probable that the hemoglobin concentration within the muscle may be a factor in determining rate of rancidification of meat. Comparisons of rancidity development in light and dark muscle from the same animal would be of interest.

*Effect of Cyanide*—The lipoxidase activities of muscle extract and hemoglobin solution with and without addition of potassium cyanide were compared in this experiment. For the control, the solution added to the lard consisted of equal parts of phosphate buffer, distilled water, and muscle extract or hemoglobin solution. In the case of the cyanide samples, the distilled water was replaced by 0.03 M KCN. The pH of the phosphate buffers was adjusted in preliminary experiments so that the final pH of the mixtures was 5.4. The results are shown in Tables I and II.

The concentrations of hemoglobin in the muscle extract and hemoglobin solution were not adjusted colorimetrically in either experiment. A more concentrated hemoglobin solution was used in the second experiment (Table II).

At pH 5.1, potassium cyanide solution added to the muscle extract or hemoglobin solution did not inhibit their lipoxidase activities. These results are in agreement with those of Robinson (6). Barron and Lyman (9) reported an inhibitory effect of KCN on hemin catalysis at a pH of 9, but the effect became vanishingly small at pH values below 7.

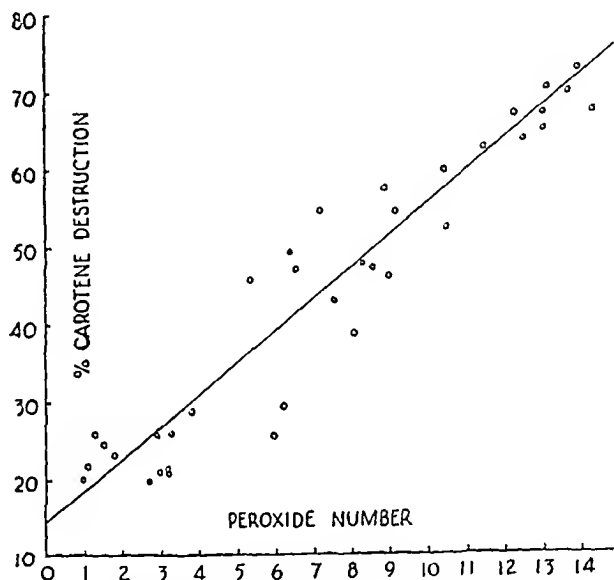


FIG. 7 Correlation of carotene destruction with peroxidation of lard catalyzed by muscle extract or hemoglobin solution

*Correlation of Carotene Destruction with Peroxide Number of Fat*—During the oxidation of unsaturated fatty acids in the presence of muscle extract or hemoglobin solution,  $\beta$ -carotene added to lard was destroyed (Fig. 7). Some destruction of carotene preceded the first detectable increase in peroxide number. At a peroxide number of 9, approximately 50 per cent of the carotene was destroyed. Sumner and Sumner demonstrated that  $\beta$ -carotene was destroyed very rapidly by the lipoxidase of soy bean meal in the presence of unsaturated fatty acids (11).

#### DISCUSSION

The evidence presented in this paper supports the view that the lipoxidase activity of pork muscle is due to its hemoglobin (or myoglobin).

content The magnitude of the catalytic effect of muscle extracts on fat peroxidation was only slightly less than that produced by the same concentration of hemoglobin at pH 5 Neither hemoglobin nor muscle extract catalysis was affected by cyanide, both catalysts were destroyed by heat While it was true that pH had very little effect on the catalytic activity of pure hemoglobin solutions, whereas the activity of muscle extracts rapidly fell to zero as the pH was increased from 5 to 7, the evidence clearly demonstrated that this difference in behavior is ascribable to inhibiting substances in the muscle extracts The same inhibitory effect of pH was obtained when blood hemoglobin was added to muscle extract

The inhibitory substances were not identified The inhibition could conceivably have been caused by phenolic and synergistic antioxidants of the type so well established by Mattill and coworkers (17) However, there seems to be no published evidence to indicate that pH changes in this range have a marked effect on the antioxidant activity of tocopherols or other inhibitors

Binding of the hemoglobin molecules by oppositely charged muscle proteins in the region of maximum inhibition would seem to be a more reasonable explanation This is supported by the observation that, at the pH of maximum precipitation of muscle proteins, inhibition of hemoglobin catalysis is at a minimum It would be of interest in this connection to free muscle extract from the bulk of the muscle proteins by precipitation at pH 5.2, and then determine the lipoxidase activity of the supernatant solution as a function of pH

Applications of these findings to the problem of rancidity in pork have already been pointed out (4) Their possible physiological significance has not been explored At the normal pH of the resting muscle, the catalytic activity of hemins was at a minimum, but even in this pH range (7.0 to 7.4) the effect might be appreciable in the more concentrated myoglobin solutions of the intact red muscle Drop in pH within the muscle after stimulation or voluntary work normally amounts to 0.1 to 0.3 pH unit, and may reach 0.7 pH under conditions in which the normal blood flow is interfered with (18)

Tocopherols and other phenolic substances capable of forming quinones have been shown to inhibit the hemoglobin-catalyzed oxidation of unsaturated fats (10) Tissues from animals deprived of tocopherol show much higher oxygen consumption than corresponding tissues from normal animals (19, 20) Ascorbic acid and other synergists reinforce the inhibiting action of tocopherols on unsaturated fat oxidations, although activity of such synergists on hemin-catalyzed oxidations of fat has apparently not been explored Increased oxygen consumption has also been reported in ascorbic acid-deficient guinea pig tissues (21)

It would be of interest to know whether the increased oxygen consumption in tissues of animals deprived of vitamins E or C can be attributed to an acceleration of the coupled oxidation between hemins and unsaturated fats. The rôle of the entire system (unsaturated fat, hemin catalyst, tocopherol, and other antioxidants) in biological oxidations is still obscure.

#### SUMMARY

1 The effect of pork muscle extract on the peroxidation of lard was compared with that of a hemoglobin solution obtained from hog blood. At pH 5, the muscle extract caused only slightly less acceleration of oxidation than a hemoglobin solution adjusted colorimetrically to the same hemin content.

2 The catalytic effects of both muscle extract and hemoglobin were destroyed by heat, decreased with dilution, and were unaffected by cyanide at pH 5.3.

3 Muscle extract contained substances which progressively inhibited the catalytic effect of native or added hemoglobin in the pH range 5 to 7.

4 The lipoxidase activity of muscle extract is believed to be due to its hemoglobin (or myoglobin) content.

5 Applications of the findings to rancidity development in pork are discussed, as well as their possible physiological significance.

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# REJUVENATION OF MUSCLE ADENYLIC ACID NITROGEN IN VIVO STUDIED WITH ISOTOPIC NITROGEN\*

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(Received for publication, June 13, 1947)

It is known that in skeletal muscle adenylic acid is deaminated to inosinic acid if the muscle is subjected to strong tetanic contractions (1-3). Inosinic compounds are subsequently reaminated to adenylic acid compounds during the aerobic recovery period (4, 5). However, it has not been possible to demonstrate deamination-reamination in the resting muscle or during moderate muscular work *in vivo*. This might be interpreted in two ways. Either deamination-reamination of adenylic acid takes place only during severe muscular work or a deamination-reamination occurs under all circumstances, but the rate of the reamination, being higher than that of deamination, bars any accumulation of deamination products.

Barnes and Schoenheimer (6) in their studies of the purines of nucleic acids and nucleotides found that, whereas the nitrogen of nucleic acid purines was undergoing a considerable rejuvenation, the nitrogen of muscle adenylic acid showed an  $N^{15}$  concentration which amounted to only 20 per cent of that of the total nitrogen (ring N as well as amino N) of the purines of nucleic acids. This could mean either that the rate of rejuvenation of adenylic acid N was only one-fifth of that of nucleic acid N or that only one of the five nitrogens of adenylic acid was undergoing rejuvenation. In order to decide between the two possibilities, techniques were developed in the present series of studies which made it possible to collect separately the 6-amino nitrogen and the ring nitrogen of muscle adenylic acid from rats fed  $N^{15}$ , and thus to differentiate between the  $N^{15}$  concentration of the two sources of nitrogen. The  $N^{15}$  was administered as ammonium citrate by stomach tube and the animals were sacrificed after 7 to 8 hours (for details, see "Experimental"). Adenyl pyrophosphate was isolated from the muscles and purified as crystalline adenylic acid. The 6-amino N was split off by means of the specific adenylic acid deaminase (7, 8). The ring nitrogen (inosinic acid) and the ammonia derived from the 6-amino group were analyzed separately for  $N^{15}$ . An  $N^{15}$  analysis of the nitrogen of

\* This work was in part supported by grants from the Josiah Macy, Jr., Foundation and the Nutrition Foundation, Inc.

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adenylic acid was also made in order to obtain a value of 6-amino nitrogen plus ring nitrogen combined. The enzymatic deamination not only insures greater specificity but also permits direct isotope determinations of the amino N, whereas deamination by nitrite would release the amino N as molecular nitrogen which would be lost.

The isotope concentrations of the various N fractions are given in Table I.

It can be seen that the isotopic nitrogen of muscle adenylic acid is confined to the 6-amino group with little or no  $N^{15}$  in the ring nitrogen. The  $N^{15}$  concentration of the adenine N as calculated from the  $N^{15}$  values of 6-amino N and the ring N would be 0.019 to 0.020 atom per cent excess, which is in good agreement with the value 0.021 atom per cent excess actually found.

These results indicate that the adenylic acid in skeletal muscle is subject, *in vivo*, to a very rapid deamination-reamination. The rate of this ammonia cycle varies probably with the degree of activity of the skeletal

TABLE I  
 *$N^{15}$  in Various Parts of Muscle Adenylic Acid after Enzymatic Deamination*

Type of nitrogen	Atom per cent excess $N^{15}$
6-Amino N	0.093
Ring N from hypoxanthine-ribose phosphate	0.007
Total N of adenylic acid	0.021

muscle. A comparison of the rates of rejuvenation of the 6-amino N in resting muscle (for instance a paralyzed hind leg) and in the contracting muscle would undoubtedly be of interest. The muscle investigated in the present series of studies must be considered as being in a state of fairly high activity.

Inasmuch as studies of the nitrogen metabolism *in vivo*, with  $N^{15}$  as tracer, so far have been performed over a longer period of time (24 to 72 hour feeding experiments), it was considered of interest to determine the  $N^{15}$  concentration in a number of other nitrogenous compounds in muscle.

The purines of the nucleic acids of skeletal muscle were isolated according to the method of Graff and Maculla (9) and the nitrogen subjected to isotope analysis. It was found in one experiment that the excess  $N^{15}$  concentration of the nucleic acid purines amounted to 0.030 atom per cent after 7 to 8 hours. The location of the  $N^{15}$  in these compounds was not determined, since the amount of nucleic acid obtained from skeletal muscle was not sufficient to permit any fractionation. It will be recalled that



Barnes and Schoenheimer (6) found that the  $N^{15}$  in the liver nucleic acid seemed to be fairly equally distributed between the amino N and the ring N of the purines

The amide nitrogen and glutamic acid nitrogen of the muscle proteins were also subjected to an  $N^{15}$  analysis. The  $N^{15}$  concentration of the amide was high (0.100 atom per cent excess). The  $N^{15}$  concentration of the glutamic acid amounted only to 0.020 atom per cent excess.

It is of interest that the glutamic acid N which is considered to have a relatively high rate of rejuvenation, even in muscle, in an 8 hour experiment attained an isotopic concentration which was only about one-fifth of that of the 6-amino N of muscle adenylic acid. The amide N, on the other hand, attained an  $N^{15}$  concentration even higher than that of the 6-amino N of the adenylic acid.

The mechanism of reamination of inosinic acid to adenylic acid is not known as yet. The studies presented here render it improbable that glutamic acid directly reaminates inosinic acid. The high  $N^{15}$  concentration of the amide N suggests amides as a possible source of nitrogen for this reamination. However, no definite conclusions regarding the pathway of the ammonia cycle in muscle are possible at the present time.

#### EXPERIMENTAL

Three male rats weighing 350 to 400 gm. were fed 2.4 gm. of ammonium citrate containing 33 atom per cent excess  $N^{15}$ . The ammonium citrate solution was administered by stomach tube in three equal portions at 2 hour intervals. The rats were sacrificed 8 hours after the first administration of  $N^{15}$ . The carcasses were chilled in ice and the skeletal muscles of the three rats were pooled (weight approximately 200 gm.). Adenyl pyrophosphate was isolated according to Lohmann's method and the pure adenyl pyrophosphate was subjected to dephosphorylation of the labile groups in baryta. The adenylic acid was precipitated with lead acetate and finally recrystallized twice as the free acid from aqueous solution. The crystalline adenylic acid was dried at  $80^\circ$  *in vacuo*, yield, 70.2 mg.

*Analysis of Crystalline Adenylic Acids*—Total N (Kjeldahl, 18 hours) 20.3 per cent, theory 20.1, total P (no inorganic P) 9.08 per cent, theory 8.94, ribose 43.8 per cent, theory 43.2, molecular  $E_{240} = 1.6 \times 10^4$ , theory  $1.5 \times 10^4$ .

A solution of this material, treated with muscle deaminase, gave in the ultraviolet spectrophotometer (cf. (8))  $-\Delta \log (i_0/i)_{\%} = 0.208$  for 10  $\gamma$  of nucleotide per ml., theory 0.196.

$N^{15}$  concentration was 0.021 atom per cent excess.

*Enzymatic Deamination of 5-Adenylic Acid to 5-Inosinic Acid*—A solution of 28 mg. of adenylic acid in 4 ml. of water was neutralized with N NaOH

to pH 6, and 0.5 ml of 0.7 M potassium acetate (pH 6) was added, followed by 2 ml of dialyzed muscle deaminase, blank, enzyme mixture without adenylic acid. Aliquots of 50 c mm were drawn from the adenylic acid mixture and from the blank at the start of the experiment and after 2 and 4 hours incubation at 37°, and the ultraviolet spectrum was determined. The absorption of the enzyme was subtracted. When the adenylic acid mixture had attained a pure inosinic acid spectrum (cf (8)) the reaction was stopped with 1 ml of 20 per cent trichloroacetic acid. Inosinic acid was precipitated with lead acetate, the blank was treated likewise, although very little precipitate developed. The supernatant was used for analysis of the 6-amino group. The solution of free acid was analyzed for P and N. N/P, found 2.05, theory 1.83.

The inosinic acid fraction was free of adenylic acid. This appears not only from the ultraviolet spectrum but also from the fact that addition of an active muscle deaminase to an aliquot of the inosinic acid solution did not cause any decrease in the density at 265 m $\mu$ .

The N<sup>15</sup> concentration was 0.007 atom per cent excess.

*6-Amino Nitrogen*—The mother liquors and washings from the lead precipitation were treated with H<sub>2</sub>S. The lead sulfide was removed by filtration and washed twice with water. The filtrate and washings were acidified and aerated, then made alkaline and steam-distilled. The ammonia derived from the deaminase preparation constituted only about 1 per cent of that of the 6-amino group and did not contain any excess N<sup>15</sup>.

The N<sup>15</sup> concentration of the 6-amino group was 0.093 atom per cent excess.

*Analysis of Purines of Nucleic Acid from Muscle*—Purines were isolated as copper salts, according to the method of Graff and Maculla (9).

An aliquot was freed of Cu by treatment with H<sub>2</sub>S, the sulfide washed several times with hot water, and the filtrate analyzed for guanine by means of guanase at 290 m $\mu$  (10).

The amount of pure N in the whole sample calculated from this analysis was 3.1 mg, found, by the direct Kjeldahl method, 3.7 mg. The 0.6 mg excess is presumably derived from the pyrimidine compounds, which are to some extent hydrolyzed by this procedure.

The N<sup>15</sup> concentration was 0.030 atom per cent excess.

*Amide N*—A 3 gm sample of acid-insoluble muscle residue was washed twice with dilute trichloroacetic acid and hydrolyzed for 24 hours in 20 per cent HCl at 105°. Amide nitrogen was obtained by the usual procedure.

Amide N 1300  $\gamma$ , N<sup>15</sup> concentration 0.100 atom per cent excess. Glutamic acid hydrochloride was isolated by the usual procedures from 40 gm of muscle tissue, N<sup>15</sup> concentration 0.020 atom per cent excess.

## SUMMARY

1 Following the administration to rats of ammonium citrate labeled with  $N^{15}$  a high concentration of isotope is found in the 6-amino nitrogen of the adenylic acid of skeletal muscle, thus indicating a very rapid, reversible deamination.

2 Under the same conditions the glutamic acid of the muscle proteins showed an isotope concentration only one-fifth of that of the 6-amino N of the adenylic acid, whereas the amide N had a higher  $N^{15}$  value. Amides are suggested as a possible source of nitrogen for this rapid rejuvenation.

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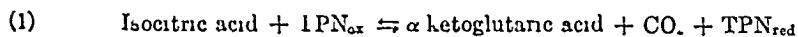
# CARBON DIOXIDE FIXATION IN ISOCITRIC ACID\*

By SANTIAGO GRISOLIA AND BIRGIT VENNESLAND

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(Received for publication, June 17, 1947)

Ochoa (1, 2) has demonstrated the occurrence in pig heart of an enzyme capable of the reversible decarboxylation of oxalosuccinic acid. When isocitric dehydrogenase is also present the enzyme system can catalyze the net reaction



Evidence supporting the existence of this reaction was obtained spectrophotometrically, by measuring the light absorption of reduced triphosphopyridine nucleotide (TPN) at  $340 \text{ m}\mu$ . Ochoa has also coupled the reaction with the glucose-6-phosphate dehydrogenase system, demonstrating the formation of isocitric acid from  $\alpha$ -ketoglutarate,  $\text{CO}_2$ , and reduced TPN. In the presence of aconitase, citric acid is formed. Using the conditions recommended by Ochoa,<sup>1</sup> we have been able to confirm these results.

Indirect evidence for the existence in pigeon liver of an oxalosuccinic carboxylase had previously been obtained by Mouldey, Vennesland, and Evans (3). Ochoa<sup>1</sup> has confirmed these findings by testing the decarboxylation of oxalosuccinic acid directly. The enzyme in pigeon liver, like that in pig heart is also associated with an isocitric dehydrogenase. It seemed desirable to obtain a confirmation of the reversibility of reaction (1) by an independent method, and this has accordingly been done with isotopic carbon. This paper presents evidence that extracts of pigeon liver acetone powder cause the incorporation of  $\text{C}^{14}$  (added as bicarbonate) into the  $\beta$ -carboxyl group of isocitric acid when  $\alpha$ -ketoglutarate and isocitrate are incubated together. There is no detectable net chemical reaction between these two substances.

## EXPERIMENTAL

### Methods

The pigeon liver enzyme used was the dialyzed acetone powder extract described by Evans, Vennesland, and Slotin (4). Isocitric dehydrogenase was the type of preparation designated "enzyme C" by Adler *et al.* (5).

\* This work was supported in part by grants from the John and Mary R. Markle Foundation and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

<sup>1</sup> Ochoa, S., personal communication.

Aconitase was prepared by ammonium sulfate precipitation of pig heart extracts<sup>1</sup> TPN of 80 per cent purity was prepared by a modification<sup>2</sup> of the method of Warburg, Christian, and Grøse (6), the yellow enzyme by the method of Warburg and Christian (7)<sup>3</sup> *dl*-Isocitric acid was synthesized according to Pucher and Vickery (8)  $\alpha$ -Ketoglutaric acid was prepared according to Neuberg and Ringer (9)

The measurement of C<sup>14</sup> activity was done essentially according to the procedure recommended by Reid (10) with a thin mica window counter For accurate determination of activity, samples of CO<sub>2</sub> were collected in CO<sub>2</sub>-free NaOH, and converted to BaCO<sub>3</sub>, which was washed well, suspended in the minimum necessary volume of ethanol, and transferred to aluminum disk containers of known diameter Evaporation of the alcohol resulted in a uniform layer of precipitate, the thickness of which was determined from its weight and the area of the disk After correction for self-absorption, such samples gave readily reproducible counts proportional to the specific activity of the carbonate Results are expressed as per cent of the specific activity of the bicarbonate of the medium at the end of the reaction

For qualitative determinations of the presence of C<sup>14</sup>, some preparations were counted directly as dried films Such determinations were not as accurate as those made on BaCO<sub>3</sub> precipitates, but served a useful purpose for orientation studies, since the presence or absence of C<sup>14</sup> in a particular fraction could thus readily be determined without conversion of the substance to CO<sub>2</sub>

*Incorporation of C<sup>14</sup>O<sub>2</sub> into Isocitrate*—The enzyme reaction mixture contained the following components 0.013 M *dl*-isocitrate, 0.033 M  $\alpha$ -ketoglutarate, 0.0007 M MnCl<sub>2</sub>, 0.005 M NaHC<sup>14</sup>O<sub>3</sub>, 150  $\gamma$  of TPN, and 4.0 ml of pigeon liver extract The total volume of the reaction mixture was 15.0 ml The pH of the medium was 7.4, the buffer being furnished by the 0.025 M phosphate of the enzyme The mixture was incubated at 40° for 2 hours in the absence of a gas phase CO<sub>2</sub> determinations made at the beginning and at the end of the incubation indicated that no appreciable formation of CO<sub>2</sub> occurred The reaction was stopped by the addition of 5.0 ml of 10 per cent metaphosphoric acid The C<sup>14</sup>O<sub>2</sub> liberated on addition of acid was collected in alkali and precipitated as BaCO<sub>3</sub> for determination of radioactivity The last traces of C<sup>14</sup>O<sub>2</sub> were washed out with CO<sub>2</sub> gas The protein precipitate was centrifuged off, and the supernatant was extracted with ether in a continuous extractor until all the organic acids had been removed from the aqueous portion The ether was evaporated and the

<sup>1</sup> Altman, K. I., unpublished method

<sup>2</sup> We are grateful for the generous cooperation of Mr. K. Altman who supplied this preparation

residue neutralized and brought to a volume of 2.0 ml. This residue consisted mainly of a mixture of isocitrate, citrate, and  $\alpha$ -ketoglutarate. The citrate was formed from the isocitrate by the action of aconitase present in the pigeon liver enzyme. Evaporation of an aliquot to a dry film and determination of radioactivity directly indicated that considerable  $\text{CO}_2$  fixation had occurred. The specific activity of the film was about 10 per cent of the specific activity of the  $\text{BaCO}_3$  prepared from the bicarbonate of the medium.

To localize the position of this fixed  $\text{C}^{14}$ , an aliquot of the ether extract was precipitated with 2,4-dinitrophenylhydrazine. 21.5 mg of the 2,4-dinitrophenylhydrazone of  $\alpha$ -ketoglutaric acid contained no appreciable radioactivity, indicating that the  $\text{C}^{14}$  content of  $\alpha$ -ketoglutaric acid was negligible. It was estimated that if 1 carbon atom in  $\alpha$ -ketoglutarate contained 0.5 per cent of the activity of the bicarbonate of the medium it could readily have been detected. The conversion of the  $\alpha$ -ketoglutarate to  $\text{CO}_2$  would not give a method of detection much more sensitive than the direct count of the precipitate.

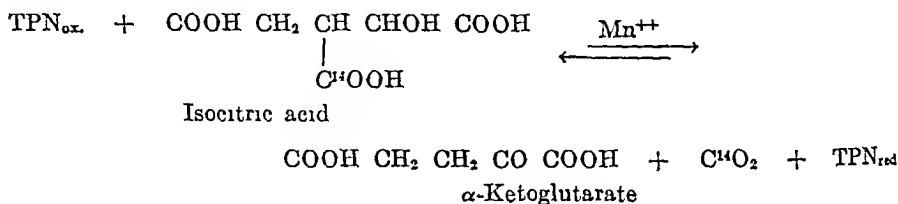
The remainder of the ether extract was treated with isocitric dehydrogenase in the presence of  $\text{Mn}^{++}$ , TPN, yellow enzyme, methylene blue, and  $\text{O}_2$  to remove the  $\beta$ -carboxyl carbon of the isocitric acid. The procedure was essentially that described by Adler *et al.* (5). Aconitase was added to insure the conversion of all citrate to isocitrate. The reaction mixture was made up to contain 0.0005  $\text{M}$   $\text{MnCl}_2$ , 0.005  $\text{M}$  veronal buffer, pH 7.4, and 0.0001  $\text{M}$  methylene blue. 15 mg of yellow enzyme of 10 per cent purity, 0.4 ml of aconitase, 2.0 ml of isocitric dehydrogenase, and 150  $\gamma$  of TPN were made up with the sample to a total volume of 10.6 ml. The mixture was incubated for 5 hours at room temperature in a continuous stream of  $\text{CO}_2$ -free air. The air was subsequently passed through alkali to absorb the  $\text{CO}_2$  which had been formed during the reaction. At the end of the reaction acid was added to insure the complete liberation of the  $\text{CO}_2$ .

Under the conditions of this reaction the isocitric and citric acids present in the sample were decomposed to give  $\text{CO}_2$  and  $\alpha$ -ketoglutaric acid. The  $\text{CO}_2$  so formed must originate from the  $\beta$ -carboxyl carbon. However, on prolonged incubation  $\alpha$ -ketoglutarate may also yield  $\text{CO}_2$  as a result of a secondary oxidative decarboxylation occurring in the presence of  $\text{Mn}^{++}$ . The amount of  $\text{CO}_2$  formed from  $\alpha$ -ketoglutarate and from isocitrate (plus citrate) respectively may be determined by chemical analysis of the isocitrate, citrate, and  $\alpha$ -ketoglutarate present at the beginning and the end of the reaction. Such determinations were in agreement with the amount of  $\text{CO}_2$  evolved. Since the  $\alpha$ -ketoglutarate contains no  $\text{C}^{14}$ , corrections may be applied for the dilution of the  $\text{CO}_2$  from the isocitrate by inactive carbon from the  $\alpha$ -ketoglutarate. After the application of such corrections, the

CO<sub>2</sub> from the isocitrate and citrate was found to contain 70 per cent of the C<sup>14</sup> activity of the bicarbonate of the medium. This indicated that an extensive exchange had occurred between the bicarbonate of the medium and the  $\beta$ -carboxyl carbon of isocitric acid.

### DISCUSSION

The exchange reaction demonstrated in these studies may be formulated as follows:



The reaction is analogous to the incorporation of isotopic carbon into the carboxyl groups of the dicarboxylic acids when pyruvate and fumarate are incubated with pigeon liver in the presence of isotopic bicarbonate (11). There are two differences which must be borne in mind when comparing the reactions, however. First, the action of aconitase in catalyzing the interconversion of isocitrate, citrate, and *cis*-aconitate does not cause a distribution of the label present in the  $\beta$ -carboxyl carbon into the other carbon atoms of the tricarboxylic acids. Consequently,  $\alpha$ -ketoglutaric acid formed from the isocitrate, is not labeled. The fixed CO<sub>2</sub> incorporated into the  $\beta$ -carboxyl carbon of isocitrate by this reaction will consequently not be distributed into the other components of the tricarboxylic acid cycle, unless citrate and isocitrate are metabolized over some other route than that involving  $\alpha$ -ketoglutarate formation. This is in contrast to the pyruvate-malate-fumarate system in which the action of fumarase results in the equilibration of the bicarbonate with all the carboxyl groups of the organic acids involved (pyruvate, lactate, fumarate, and malate) (4, 11). Second, there is little, if any, oxidation-reduction between  $\alpha$ -ketoglutarate and isocitrate in pigeon liver extracts. The fixation of CO<sub>2</sub> occurs in the absence of any net reaction between the components of the system. In the case of the pyruvate-malate system in pigeon liver, the fixation is accompanied by an oxidation-reduction between pyruvate and malate, resulting in a net conversion of the malate to lactate and CO<sub>2</sub>. This is due to the presence of a lactic dehydrogenase. A corresponding enzyme capable of reducing  $\alpha$ -ketoglutaric acid is absent. It is consequently apparent that the oxidation-reduction of the  $\alpha$ -keto acid is not necessary for the fixation in reactions of this type.



## CHROMOGENIC SUBSTRATES

### IV ACYL ESTERS OF *p*-NITROPHENOL AS SUBSTRATES FOR THE COLOIMETRIC DETERMINATION OF ESTERASE\*

By CHARLES HUGGINS AND JACK LAPIDES

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(Received for publication, June 18, 1947)

The development of color from the decomposition of colorless esters of *p*-nitrophenol by enzyme action furnishes a delicate test of esterase activity, by this means the esterase patterns of serum were investigated. In the past the detection of esterase action has been carried out by means of biologic, physical, and chemical methods. In the biologic method (1) the frog heart has been used for estimating the concentration of acetylcholine left over after hydrolysis, and the physical basis of assay has been the decrease of the viscosity of tributyrin (2) as determined by the drop count technique. These have presented obvious disadvantages, in the stalagmometric method the decrease of drop number is much greater than the percentage hydrolysis by titration (3). All of the chemical techniques have been based on the estimation of acid production by titration, measurement of pH changes, or the manometric estimation of the displacement of carbon dioxide from a bicarbonate buffer, the most satisfactory, evidently, being the technique of Ammon (4). In general some of the difficulties have been that in the titimetric method pH changes in the system due to the liberation of acid affect enzymic activity and there has been the inconvenience of the Warburg technique for routine use. Also with slightly soluble esters such as the butyrates it has been difficult to obtain a standardized emulsion, so that the reproducibility of the assay has been only about 10 per cent. Abdon and Uvnas (5) attempted to estimate acetate production from acetylcholine colorimetrically by adding ferric chloride to the mixture after long incubation, but among the difficulties in this method is the fact that color production by ferric ion is not specific for acetate.

The method described in this paper utilizes a new principle in that the velocity of enzyme action is determined by estimating the amount of the hydroxyl group which is liberated. Several esters of *p*-nitrophenol were prepared and were found to be readily hydrolyzed by esterases, liberating *p*-nitrophenol, which is readily measured colorimetrically.

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Most workers are in agreement that there exist two types of esterases, the one hydrolyzing acetylcholine, discovered by Loewi and Navratil (6) and set in a special category as cholinesterase by Stedman, Stedman, and Easson (7), the other esterase is considerably less specific and has been studied by a great number of techniques in which the substrates most commonly used have been ethyl butyrate (8), methyl butyrate (9), mandelic esters (10), and tributyrin (2). The latter esterase has received a number of designations, simply as esterase (11), the "serum enzyme of human blood" (12), the S-type (13), pseudocholinesterase (14), and unspecified esterase (15), in this paper we shall follow the terminology of Richter and Croft (16) in referring to this second activity as aliesterase, "an esterase of the type that acts preferentially on the simple aliphatic esters and glycerides."

While cholinesterase has been extensively investigated, not much is known about the aliesterases, largely because of technical difficulties in obtaining standardizable and soluble substrates, such disadvantages are absent from the present method, which accordingly permits the systematic analysis of these enzymes.

### *Synthesis of Substrates*

The following *p*-nitrophenyl esters were prepared: the acetate (PNPA), propionate (PNPP), isobutyrate, trimethyl acetate, *n*-butyrate, isovalerate, and *n*-valerate. The substrates were made according to the method of Spasov (17). *p*-Nitrophenol, 13.9 gm (0.1 mole), is mixed with 30 gm of benzene and 1.2 gm of magnesium turnings in a round bottom flask and 0.12 mole of acyl chloride is added. The mixture is refluxed for 1 hour on a steam bath and then cooled. About 150 ml of ethyl ether are added and the mixture is transferred to a separatory funnel, where it is washed successively with water, aqueous saturated sodium bicarbonate solution, and again with water, the aqueous washing solutions being discarded. The benzene-ether mixture is dried with anhydrous sodium sulfate and the solvents evaporated at room temperature.

In general it was found difficult to separate all traces of accompanying free *p*-nitrophenol from the products (Table I), although enough was removed to make them serviceable as substrates. The solid esters were partially purified by 3-fold recrystallization from about 150 ml of ethyl ether, in the case of *p*-nitrophenyl propionate the crude product was dissolved in about 20 ml of *n*-butanol at 45°, on cooling to room temperature, crystallization took place.

The liquid esters were washed three times with about 50 ml of water, which was removed following centrifugation. Finally anhydrous sodium sulfate was added to complete the dehydration.

*p*-Nitrophenyl acetate has been prepared by Noeltling, Grandmougin, and Michel (18), we have been unable to find a record of the synthesis of the other esters described in this paper. The chemical composition of the esters with respect to *p* nitrophenol content and the melting points are given in Table I. None of the liquid esters crystallized above 4°

## EXPERIMENTAL

*Substrate Concentration and Velocity of Enzyme Action*—With a saturated aqueous solution of PNPA and PNPP, esterase activity of human serum was determined against varied concentrations of substrate in three experi-

TABLE I  
Characteristics of *p*-Nitrophenyl Esters

	M p	Solubility in water at 25	Free <i>p</i> ni- trophenol associated with ester	<i>p</i> Nitrophenol yielded by ester on hydrolysis*		Remarks
				Calculated	Analyzed	
	<i>C</i>	gm per 100 gm	gm per 100 gm	gm per 100 gm	gm per 100 gm	
Acetate	77-78	0.60	2.5	76.8	77.2	Large truncated py- ramidal crystals
Propionate	62-63	0.245	2.33	71.3	72.4	Long white needles
Isobutyrate	25-27	0.232†	3.01	71.0	69.8	Short " "
Trimethyl acetate	94-95	0.0006	0	62.4	63.0	Hexagonal plates
<i>n</i> -Butyrate	‡	0.240	2.89	71.3	68.0	Brown oily liquid
<i>n</i> -Valerate	‡	0.094	2.62	66.3	64.4	" " "
Isovalerate	‡	0.089	2.01	66.6	67.0	" " "

\* The weight of free *p*-nitrophenol was subtracted from the total weight of the compound before the calculations

† Solubility at 23°

‡ Liquid at room temperature

ments. From the plot of reaction velocity against substrate content (Fig. 1) the Michaelis and Menten constants (19) were determined, for PNPA this constant was found to be  $4.15 \times 10^{-3} M$  and for PNPP  $2.5 \times 10^{-3} M$ .

All of the substrates underwent hydrolysis in *M*/15 phosphate buffer at pH 7 at 25° (Fig. 2), and with sufficiently high substrate concentration to keep the enzyme saturated, inconveniently large amounts of color were produced non-enzymically. In order to reduce the color blank it was decided to use much lower concentrations of substrate than are required to saturate the enzyme and experimental conditions were therefore determined under which a linear relationship resulted between enzyme concen-

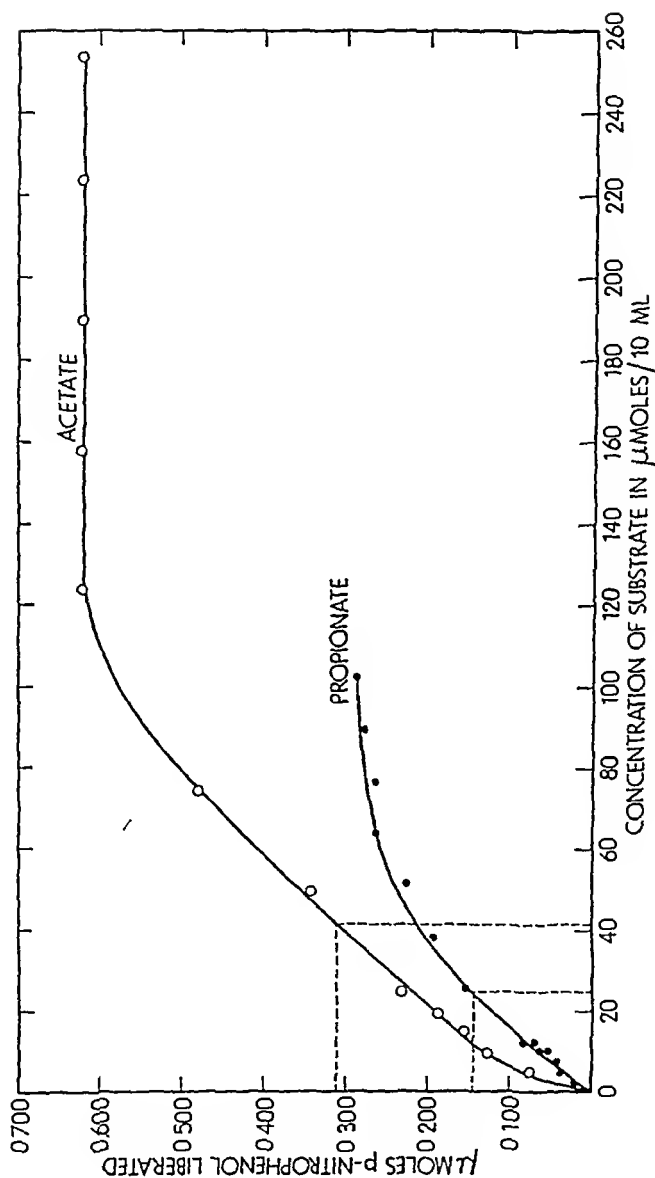


Fig. 1 The effect of substrate concentration on rate of enzymic hydrolysis of *p*-nitrophenyl acetate and propionate. The reaction rate is expressed in micromoles of *p* nitrophenol liberated in 20 minutes at 25°. The system consisted of 1 ml of 0.1 M phosphate buffer, pH 7, 0.5 ml of 4 per cent human serum, and varying amounts of saturated aqueous solutions of the substrates, water was added to make the final volume 10 ml

titution and *p*-nitrophenol liberated. It was found that this relationship held with dilutions of serum up to 25-fold when the substrate content was 0.666 micromole in a final volume of 10 ml, provided that not more than 13 per cent (0.286 micromole) of the substrate was split. Further, since the line passed through the zero point (Fig. 3), it is established that the velocity of hydrolysis is proportional to the enzyme activity. This linear relationship was found to be true for all of the nitrophenol substrates described in this paper. Van Slyke (20) has outlined the conditions necessary for measuring enzyme activity at low substrate concentration. One of these conditions is that the substrate concentration is a critical factor in the analysis and care must be taken in daily work to prepare the substrate solution accurately.

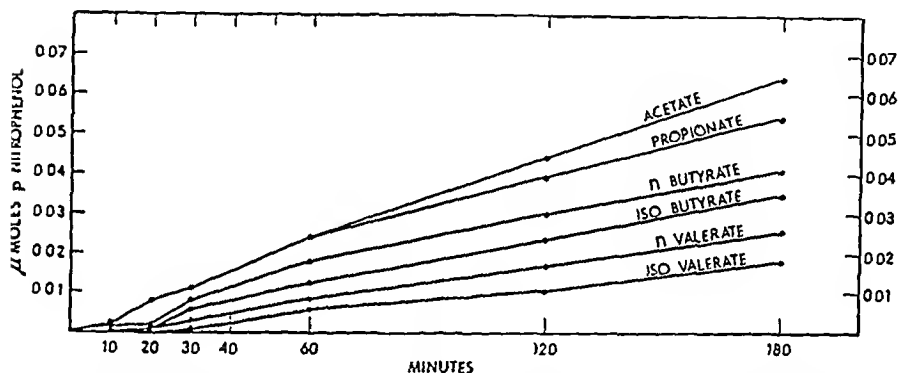


FIG. 2 The rate of hydrolysis of the *p*-nitrophenyl esters, acetate, propionate, *n* butyrate, isobutyrate, *n* valerate, and isovalerate, in 0.066 *M* phosphate buffer, pH 7. The system consisted of phosphate buffer, 2 ml, water 6 ml, and substrate 2 ml, the final concentration of substrate was 0.666 micromole.

With a substrate concentration of 0.666 micromole in 10 ml, the enzyme velocity is not proportional to time except with very dilute enzyme solutions, the curve becoming progressively depressed. Accordingly the tests must be run for an identical time for comparative results and in these experiments the standard time elected was 20 minutes.

**Temperature Effects on Esterase Action**—The optimum temperature for activity of human serum esterase against PNPP (Fig. 4) was 32°, at 25° the enzyme acts at about 87 per cent of the optimum. Earlier, the temperature optimum for pancreatic esterase (ethyl butyrate) was found to be about 30° (8). We also determined the temperature coefficient,  $Q_{10} = (\text{velocity at } T^\circ + 10) / (\text{velocity at } T^\circ)$ . Between 18–28°,  $Q_{10}$  was 1.59. Vahlquist (21) observed for acetylcholine a  $Q_{10}$  of 1.42 and for methyl butyrate the  $Q_{10}$  was 1.4, a  $Q_{10}$  of 1.7 for human serum acting on acetylcholine was obtained by the ferric chloride method (5).

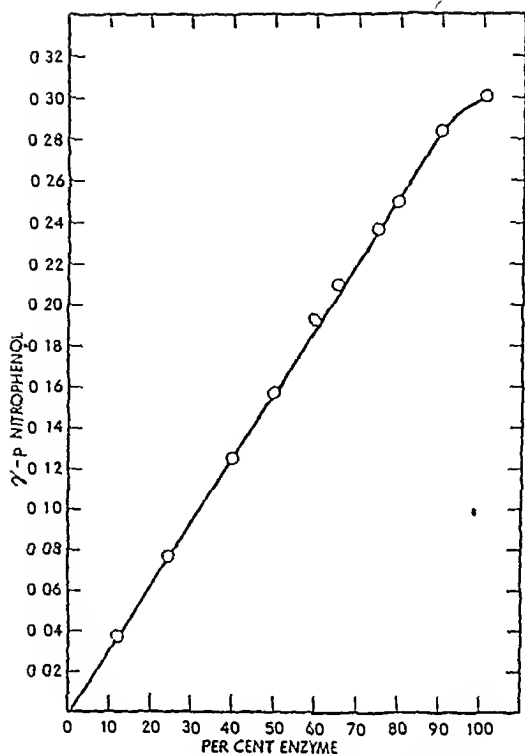


FIG 3 The effect of enzyme concentration on the rate of hydrolysis of *p* nitro phenyl propionate The system consisted of 2 ml of 0.066 M phosphate buffer, 7 ml of water, and 1 ml of various dilutions of human serum, incubation 20 minutes at 25°

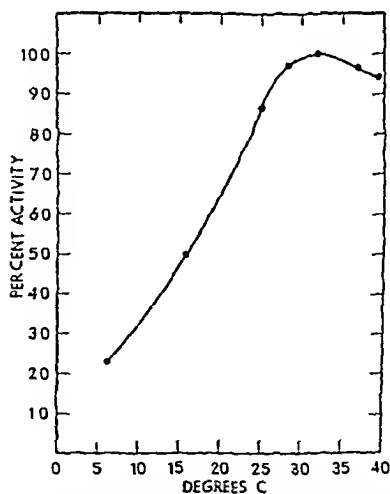


FIG 4 Esterase action of human serum on *p*-nitrophenyl propionate at varying temperatures The system consisted of 2 ml of 0.066 M phosphate buffer, 7 ml of water, 1 ml of 4 per cent human serum, and 1 ml of substrate (0.333 micromole), incubated 20 minutes



*Lipase and pH*—A series of buffers was made with 0.05 M tris(hydroxymethyl)iminomethane maleic acid<sup>1</sup> and also 0.05 M sodium barbital-hydrochloric acid (22) between pH 5.15 and 9.4. The experimental system consisted of 1 ml of buffer and 1 ml of 1 per cent serum, while in control tubes serum was replaced with water. At 1 minute intervals 1 ml of substrate was added to both sets and, after precisely 20 minutes, 7 ml of 0.2 M phosphate buffer at pH 7 were added to bring to standard pH and the tubes were read in a colorimeter. All pH estimations were determined potentiometrically in a glass electrode and the final pH of these solutions varied from 6.91 to 7.15, the free *p*-nitrophenol content was read from a

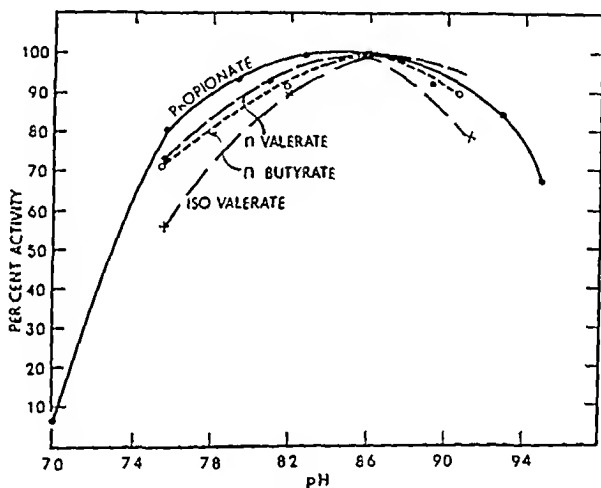


FIG 5 The effect of pH on the rate of enzymic hydrolysis of the *p*-nitrophenyl esters, propionate, *n*-butyrate, isovalerate, and *n*-valerate. The system consisted of 2 ml of 0.1 N barbital-hydrochloric acid buffers at varying pH, 1 ml of 4 per cent human serum, 5 ml of water, and 2 ml of substrate, final concentration of all substrates 0.666 micromole in 10 ml, incubated at 25° for 20 minutes.

calibration curve made at pH 7. The curves of activity of serum against pH in all of our substrates (Fig 5) were similar and optimum activity was found at pH 8.4 to 8.6. These findings agree with those of Gluck (23) who observed that the pH optimum for the splitting of acetylcholine by human serum was 8.4 to 8.5. When the system is buffered at pH 7 as in the method adopted, the enzyme activity is about 7 per cent of its maximum, the instability of the substrates at the optimum pH precluded the use in routine analyses of the optimum pH for the enzyme.

*Organic Inhibitors of Esterase*—Ethyl alcohol depressed the esterase

<sup>1</sup> We are indebted to Professor George Gomori for furnishing this buffer.

activity of human serum against PNPP more than methyl alcohol did (Fig 6) Methyl alcohol was employed in the method adopted as a solvent for the substrates but the amount (final concentration 0.02 per cent) used does not interfere with enzyme action

The effects of eserine, ethyl carbamate, and tetraethyl ammonium chloride on the hydrolysis of PNPP by human serum (Fig 7) were determined Richter and Cloft (16) found that an eserine concentration of  $10^{-5}$  M caused almost complete inhibition of hydrolysis of acetylcholine, methyl butyrate, and tributyrin by human serum, with horse serum, activity also was inhibited completely with respect to cholinesterase but

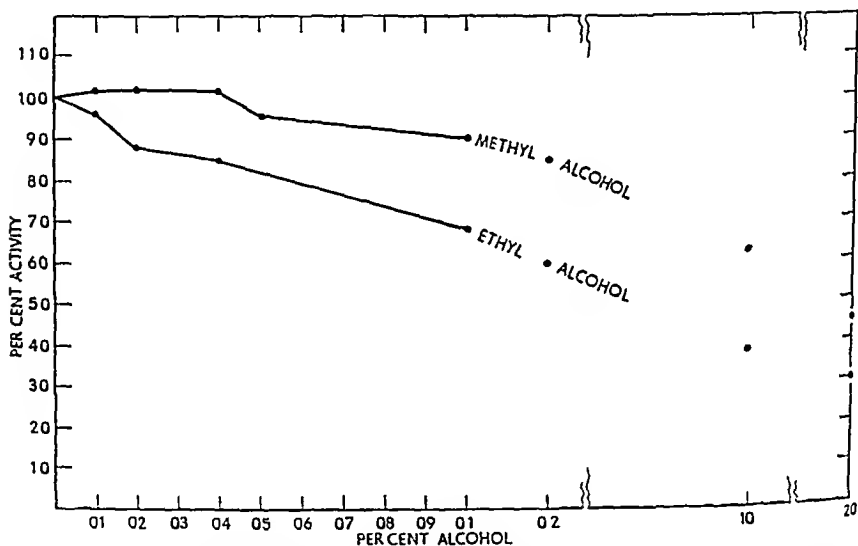


FIG 6 The effect of ethyl alcohol and methyl alcohol on enzymic hydrolysis of *p* nitrophenyl propionate The system consisted of 2 ml of substrate (0.666 micro mole), 2 ml of 0.066 M phosphate buffer, pH 7, 1 ml of 4 per cent human serum, and varying amounts of alcohol and water to make a total amount of 10 ml, incubated at 25° for 20 minutes

there was merely slight inhibition of alchelerase, we found that eserine in  $10^{-5}$  M concentration inhibited the esterase of human serum on *p* nitrophenyl esters only slightly The amount of enzyme present is a factor in eserine inhibition, in our experiments eserine  $10^{-5}$  M caused 60 per cent inhibition when 0.01 ml of rabbit serum was used and no inhibition with 0.1 ml of the same Also when eserine was in contact with the enzyme for 30 minutes prior to the test, an average of 7 per cent greater inhibition occurred than when there was no earlier association of enzyme and inhibitor, long contact of substrate and eserine did not affect the inhibiting effect of eserine

*Salt Effects on Esterase*—Human and rabbit sera were dialyzed against water at 1° for 5 days and the esterase activity on acetylcholine and PNPP was determined in the presence of concentrations of sodium chloride and potassium chloride between  $10^{-4}$  and 2 M. In the assay of cholinesterase the gasometric method of Ammon (1) and the technique of Mendel and Mundell (21) were used, except that the experiments were run for 20 minutes after tipping acetylcholine bromide from the side arm of the Warburg vessel into the main compartment.

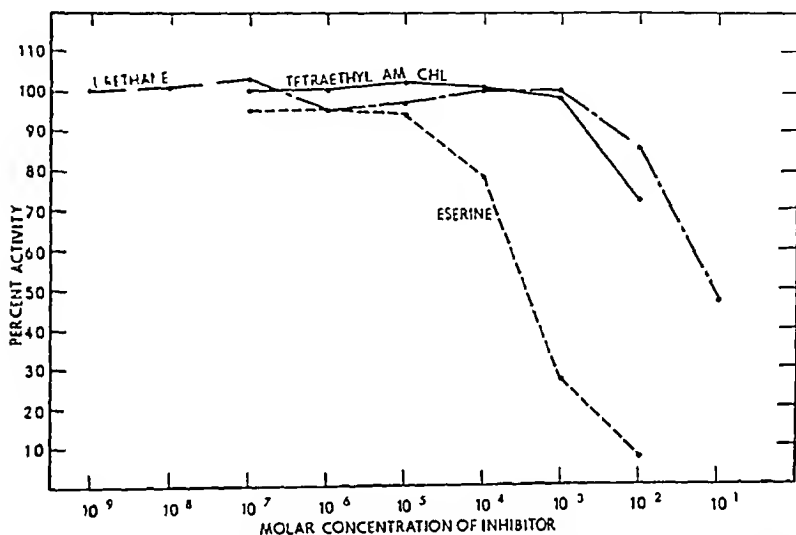


FIG. 7 The effect of ethyl carbamate, tetraethyl ammonium chloride, and physostigmine salicylate (eserine) on the rate of hydrolysis of *p* nitrophenyl propionate by human serum. The system consisted of 2 ml of substrate (0.666 micromole), 2 ml of 0.066 M phosphate buffer, pH 7, 1 ml of 4 per cent human serum, 4 ml of water, and 1 ml of various concentrations of inhibitors to give the final concentration indicated on the abscissa, incubated 20 minutes at 25°.

The action of equimolar solutions of sodium and potassium chlorides on PNPP was identical (Fig. 8), producing at first a slight acceleration with salt concentration of  $10^{-4}$  to  $10^{-2}$  M, followed by a profound depression. With respect to the splitting of acetylcholine by serum, 0.125 M sodium chloride caused 27 to 72 per cent acceleration and in 1 M concentration either no or very slight inhibition.

These findings confirm the observation of Glick (25) who showed that sodium and potassium chlorides caused activation of esterase of dialyzed rabbit serum on acetylcholine as contrasted with horse serum. However, Glick observed that there was increased acceleration with increasing salt

concentration as far as 3 M, whereas we found that above 1 M salt content inhibition set in

*On the Type of Esterase*—The activities of ten human sera were in fair agreement when PNPP and acetylcholine were used as substrates. There was a lack of close correlation between the splitting of PNPP with either methyl butyrate or tributyrin.

In the serum of the rabbit extremely high activity was found against the *p*-nitrophenyl esters and very low activity against acetylcholine, in 20

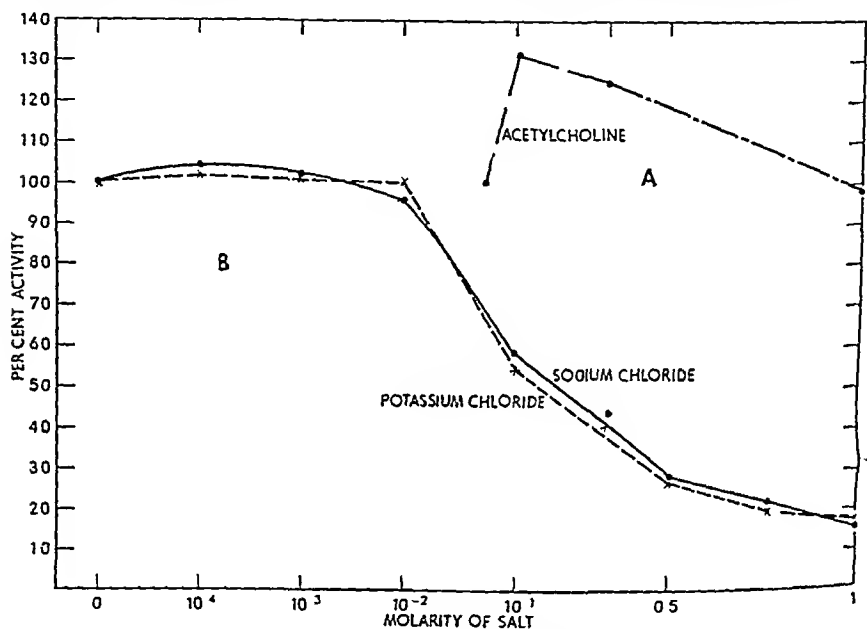


FIG. 8 The effect of various concentrations of salt on the hydrolysis of acetylcholine and *p*-nitrophenyl propionate by dialyzed human serum. The activity is stated with reference to the salt-free serum, which is assigned a value of 100 per cent. Curve A shows the effect of sodium chloride on the activity of human serum in splitting acetylcholine. Curves B show the inhibition of serum esterase by sodium chloride and potassium chloride. The molarity given on the abscissa is the final concentration of salt. Incubated 30 minutes at 25°.

minutes in Experiment 1 these values for PNPP and PNPA respectively were 31.6 and 17.4 micromoles per 1 ml, while only 5 micromoles of acetylcholine were hydrolyzed.

A concentrated extract of the electric organ<sup>2</sup> of *Electrophorus electricus* was tested against acetylcholine and PNPP, the extract was diluted 1:600 before the assay of cholinesterase. It was found that the extract split

<sup>2</sup> We are indebted to Professor David Nachmansohn of Columbia University for the gift of this material.

acetylcholine at the rate of 600,000 micromoles and PNPP 1 micromole per 1 ml, each in 20 minutes

The presence of cholinesterase in human red blood cells was demonstrated. Because of the depth of color of hemoglobin, it was necessary to use a strong light source, and in this technique a 12 volt battery was used in the photoelectric colorimeter. 1 ml of washed, packed red blood cells was hemolyzed in 99 ml of water. 1 ml of the laked blood was added to the buffered substrate ind, with the tube in place, the galvanometer was adjusted to 100. The experimental tube was then replaced by a tube containing 0.5 micromole of *p*-nitrophenol in 10 ml of alkali and a reading obtained, after the incubation period of the experimental tube was completed, the tube was read with the galvanometer adjusted to this setting. The activity of the red blood cells against PNPP in three experiments amounted to 33 to 38 per cent of the serum at the same dilution, an appreciable content of cholinesterase activity, in confirmation of Richter and Croft (16).

### Method

#### Reagents—

*M/15 phosphate buffer, pH 7.0*

*0.1 N sodium hydroxide*

*p*-Nitrophenol standards. Prepare solutions containing 10 to 70 micromoles per liter of *p*-nitrophenol, mol wt 139.1

*Substrate*—Depending on the substrate required, the following esters of *p*-nitrophenol are dissolved in 10 ml of methyl alcohol respectively as stock solutions. PNPA about 63 mg, PNPP about 65 mg, isobutyrate about 71 mg, trimethyl acetate about 79 mg. For the esters *n*-butyrate, isovalerate, or *n*-valerate, 0.5 ml is dissolved in 100 ml of methyl alcohol. These solutions may be stored in a refrigerator for 3 or 4 days, of the stock methyl alcohol solutions 1 ml of PNPA, PNPP, or isobutyrate, or 0.67 ml of *n*-butyrate, 0.71 ml of isovalerate, or 1.0 ml of *n*-valerate is diluted to 100 ml with water. *In diluting the stock methanol solutions in water, the tip of the pipette containing the substrate is submerged in the water and the volumetric flask is whirled, otherwise precipitation will occur.* These directions give approximately correct substrate values which are slightly stronger than required. A slight precipitation usually takes place with the *n*-valeryl and trimethyl acetyl esters, which is removed by filtration.

*Technique*—In order to determine the substrate concentration conveniently, standard curves relating optical density to concentration of *p*-nitrophenol are constructed both for solutions containing 1 ml of the nitrophenol standards and (a) 9 ml of *M/15* phosphate, pH 7, and (b) 9 ml of 0.1 N sodium hydroxide. The colorimeter readings are made

immediately. The galvanometer readings of color intensity are plotted on the logarithmic scale against the content of *p*-nitrophenol in micrograms per 10 ml on the arithmetic scale of semilog paper.

Just before the enzyme assay is performed, the substrate concentration is adjusted accurately to 0.333 micromole per ml, colorimeter readings are made on solutions of 1 ml of the approximately correct substrate solution pipetted into 9 ml of phosphate buffer, which gives the amount of free *p*-nitrophenol in the compound, and also on 1 ml of substrate added to 9 ml of sodium hydroxide. In 0.1 N sodium hydroxide the substrate hydrolyzes practically instantaneously. A simple calculation then permits the substrate solution to be made up precisely.

$$\frac{(\text{Micromoles total } p\text{-nitrophenol}) - (\text{micromoles free } p\text{-nitrophenol}) \times 98}{0.333} = X$$

$X - 98 = \text{ml of water to be added to give a concentration of } 0.333 \text{ micromole per } 1 \text{ ml of dilute substrate.}$  The figure 98 represents the original 100 ml volume of dilute substrate minus 2 ml removed for the tests.

An Evelyn photoelectric colorimeter with a 400 mμ filter and with matched colorimeter tubes was used throughout. In running the test 1 ml of the liquid to be assayed is mixed with 2 ml of M/15 phosphate buffer and 5 ml of water and allowed to come to temperature in a water bath at 25°. At precise time intervals, usually 1 minute, 2 ml of the accurately adjusted substrate is added, the tube is placed in the colorimeter and the galvanometer is set at 100, the galvanometer reading when the tube is removed is noted, the "an blank." At 20 minutes the galvanometer is again set at this reading, the tube is inserted, and the final reading obtained. From the standard curve of *p*-nitrophenol in phosphate buffer the amount of *p*-nitrophenol in micromoles is read and this value minus the blank control for non-enzymic hydrolysis is multiplied by the dilution factor.

In the assay of human and rat serum 1 ml is diluted to 25 ml with water, for rabbit serum 1 ml is diluted to 100 ml.

1 unit of esterase activity is defined as that amount of enzyme liberating 1 micromole of *p*-nitrophenol in 20 minutes at 25° and pH 7 in phosphate buffer when the substrate is at a concentration of 0.666 micromole per 10 ml, provided that not more than 40 per cent of the substrate is hydrolyzed. The units are expressed per 1 ml of the undiluted fluid or 1 gm of tissue.

### Results

*Human Sera*—167 sera were assayed in duplicate. The range was from 1.5 to 10.5 units per 1 ml of undiluted serum, the average error of duplicates in this series was  $\pm 1.3$  per cent.

*Rates and Patterns of Esterase Action*—In nine cases each of normal milk

human and rat serum, the esterase activity was tested simultaneously both at pH 7 and 8.5 against equimolecular solutions of the following esters of *p*-nitrophenol acetate, propionate, isobutyrate, trimethyl acetate, *n*-butyrate, isovalerate, and *n*-valerate. When the rates of splitting of the esters were expressed in percentage of that ester split at the fastest rate, consistency of results (Table II) was observed in different sera of each species. In all cases the esters were split according to a common pattern with respect to the acyl radicals, in which acetate, propionate, and isobutyrate maintained a fixed relationship to each other, while *n*-butyrate, isovalerate, and *n*-valerate could also be grouped together. Components of each group were hydrolyzed in a fairly close ratio to their congeners, establishing a pattern. The relative rate of splitting of the two groups was affected considerably by pH and by species differences but the patterns in the groups under all circumstances were preserved.

#### DISCUSSION

Aside from advantages of simplicity and accuracy, the chromogenic substrates possess the advantage of great sensitivity in the assay of esterase. Vahlquist (21) was unable to demonstrate esterase in human cerebrospinal fluid, with our method in four instances 0.25 to 0.35 unit per 1 ml was observed.

Our findings obtained with an extract of the electric organ of the eel and the serum of rabbits are in agreement with the opinion of most workers that there are at least two types of esterases, cholinesterase and alcholinesterase. In rabbit serum a high alcholinesterase activity was associated with low cholinesterase action, while in the extract of the eel electric organ a very high content of cholinesterase was accompanied by insignificantly low splitting of PNPP. The *p*-nitrophenol substrates are preferentially split by alcholinesterase.

The enzymic hydrolysis of esters of the lower fatty acids has long attracted attention. Kastle (9) showed that varying the alkyl group combined with a lower fatty acid exerted no influence on liver "lipase," but that varying the acid combined with a given alcohol caused great variations in the stability of these esters towards the enzyme, he observed that liver extracts hydrolyzed, in descending order, methyl esters of butyric, propionic, and acetic acids. Loevenhart (26) stated that pig liver extracts hydrolyzed ethyl propionate more rapidly than ethyl butyrate, whereas with pancreatic extracts the reverse was true, these differences were not confirmed by Balls and Matlack (27), but, like Kastle (9), they observed that a change in the alcohol component of the ester did not materially affect the rate of hydrolysis except where the configuration of the carbon atom bearing the hydroxyl group is concerned, the splitting of esters of secondary and tertiary alcohols occurs only slowly. Dakin (10) found

TABLE II

*Activity of Serum in Hydrolyzing p-Nitrophenyl Esters*

Values expressed as per cent of ester split maximally

Determination No	Acetate	Propionate	Isobutyrate	Trimethyl acetate	n Butyrate	Isovalerate	n Valerate
Human serum, pH 7							
1	84.3	100	94.7	0.01	48.7	26.0	58.4
2	89.4	100	98.0	0.005	59.4	28.8	66.2
3	84.2	100	95.2	0.001	42.5	22.9	83.6
4	84.0	100	92.0	0	55.3	25.4	85.6
5	85.0	100			55.0	29.4	
6	80.2	100			52.3	27.5	
7	81.0	100			55.4	29.3	
8	91.0	100			53.2	33.0	
9	86.0	100			56.2	27.2	
Average	85±3.2S	100	94.9	0.004	53.1±4.6	27.7±2.7	73.5
Human serum, pH 8.5							
1	73.5	82.2	84.2	15.8	74.0	50.8	100
2	88.0	100	99.0	14.6	78.4	38.1	100
3	78.2	93	90.4	14.9	76.0	47.7	100
Average	79.9	91.8	91.2	15.1	76.1	45.5	100
Rat serum, pH 7							
1	30.0	62.8	37.8	35.7	100	68.0	97.0
2	27.3	58.7	35.7	31.8	100	61.6	100
3	50.4	88.0		33.7	100	52.5	98.5
4	46.3	77.8			100	55.5	
5	35.2	62.9			100	53.4	
6	39.4	71.5			100	75.2	
7	41.1	76.0			100	70.0	
8	38.3	74.5			100	67.5	
9	33.3	62.0			100	68.0	
Average	37.9±7.01	70.5±10.5	36.7	33.7	100	63.5±7.8	98.5
Rat serum, pH 8.5							
1	20.3	25	12.85	14.3	64	37.1	100
2	17.8	27.5	16.0	12.3	73.4	41	100
Average	19.05	26.25	14.42	13.3	68.7	39.05	100

that an extract of hog liver preferentially split the dextrorotatory component from racemic esters of mandelic acid, this problem of optical



preference of esterase activity (28) has been systematically studied and differences found with respect to organs and species

Stedman, Stedman, and White (11) found that serum of various animals is able to hydrolyze butyrylcholine approximately twice as rapidly as acetylcholine Glick (29) using horse serum found that the enzymic hydrolysis occurred in choline esters in the following ratios *n*-butyryl 510, isobutyryl 210, propionyl 325, acetyl 225 The experiments of Nachmansohn and Rothenberg (30) were confirmatory, they found that human serum split choline esters in the following descending order butyrylcholine, propionylcholine, and acetylcholine

With the *p*-nitrophenyl esters studied in the present paper, the patterns of enzymic hydrolysis were highly dependent on species and pH effects Esters of *p*-nitrophenol were synthesized with two purposes, first to replace hydrogen on the  $\alpha$ -carbon of the acyl chain with methyl groups and secondly to lengthen the chain Six esters could be divided according to enzyme action into two groups (a) the shorter acyl chains, acetate, propionate, and isobutyrate, (b) the longer acyl groups, *n*-butyrate, isovalerate, and *n*-valerate The groups were attacked at different rates by human and rat blood, although the patterns in the groups were preserved

In human blood at pH 7 the shorter acyl esters are split faster than the longer chains, propionate being split fastest At pH 8.5, there is a relative decrease in hydrolysis of the shorter chain esters and the longer chain units are preferentially split

In rat serum, at both pH 7 and 8.5, the shorter acyl esters are split less rapidly than the longer groupings, at pH 8.5, hydrolysis of the shorter acyl esters is further depressed with reference to the activity at pH 7 At pH 7 maximal splitting occurred with *n*-butyryl and *n*-valeryl esters, while at pH 8.5 *n*-valerate was split with greatest velocity In all cases with both rat and human serum the pattern of enzymic hydrolysis differs greatly from hydroxyl ion hydrolysis (Fig. 2)

The replacement of the hydrogen atoms on the  $\alpha$ -carbon of the acetyl group by methyl groups requires special comment In the *p*-nitrophenyl acetate esters, substitution of one methyl group (propionate) and two methyl groups (isobutyrate) enhances the rate of splitting compared with PNPA, while three methyl groups (trimethyl acetate) greatly decrease the rate of enzymic attack by serum Human serum hydrolyzed the trimethyl acetate ester practically not at all at pH 7, while definite splitting occurred at pH 8.5, rat serum, on the contrary, split this ester better at pH 7 than at pH 8.5

#### SUMMARY

1 The following esters of *p*-nitrophenol were synthesized propionate, isobutyrate, trimethyl acetate, *n*-butyrate, isovalerate, and *n*-valerate

2 Conditions were established by which the content of esterase was related to the amount of *p*-nitrophenol liberated in a proportional relationship permitting the use of these substrates as assay methods

3 These substrates are preferentially split by the common alsesterase of serum in contrast to cholinesterase The enzymic hydrolysis is greatly depressed by appropriate amounts of eserine, ethyl carbamate, tetraethyl ammonium chloride, sodium chloride, and potassium chloride

4 With reference to the sera of man and rat, there is an optimum length of the acyl chain for enzymic hydrolysis which differs in the two species, and the esters are split preferentially in patterns The effect of pH on preferential splitting of the acyl esters is considerable

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# THE UTILIZATION OF LEUCINE DERIVATIVES BY A MUTANT STRAIN OF *ESCHERICHIA COLI*\*

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(Received for publication, June 27, 1947)

The ability of suitable strains of *Escherichia coli* to use certain peptides and other derivatives of phenylalanine and tyrosine as growth factors in place of the free amino acids has been reported recently from these laboratories (1). In the present communication, there are described the results of a study of the growth-promoting action of derivatives of leucine for a *leucineless* mutant strain.

The *leucineless* strain is a double mutant of strain K-12 of *Escherichia coli* and differs from the latter by its requirement for exogenous sources of threonine and leucine (2). The threonine requirement of the double mutant was induced by the irradiation of strain K-12 with x-rays (3). A second treatment of the resultant *threonineless* mutant (strain 679) with x-rays produced the double mutant (strain 679-680) requiring leucine as well as threonine (2). The experiments reported here are concerned only with the leucine requirement, and, in all the tests, the culture media contained ample DL-threonine (0.5 mg per 10 ml) to meet the requirement for that compound. In the following discussion, therefore, strain 679-680 will be considered as a *leucineless* strain.

**Testing Methods**—The methods used to study the ability of the mutant to use leucine and its derivatives were similar to those described previously (1). Tests were carried out with 10 ml of leucine-free minimal medium (3), to which threonine and the test compound were added prior to sterilization by autoclaving. After inoculation with a drop of an aqueous suspension of cells, the cultures were incubated at 30° for 24 hours. For test compounds which proved to be active, a complete growth curve with graded amounts of compound was obtained, and the molar concentration of the compound producing half maximal growth was estimated from this curve. The amount of growth was determined by density measurements in the Evelyn photoelectric colorimeter with a No. 540 filter. The relative activity of a test substance as compared to leucine was calculated from the curves for the test and reference compounds obtained on the same day.

\* These studies were aided by grants from the Williams-Waterman Fund of the Research Corporation and from the Rockefeller Foundation.

Compounds which did not replace leucine as a growth factor for the mutant were tested for possible sparing action on the leucine requirement. For these tests, the minimal medium was made up with sufficient leucine to permit approximately half maximal growth of the organism, and the growth obtained on addition of the test compound was measured as usual.

TABLE I  
*Effect of Leucine Derivatives on Growth of Leucineless Mutant Strain*

Compound	Activity as growth factor			Sparing action on leucine requirement	
		Quantity, per 10 ml. for half maximal growth	Maximal quantity per 10 ml., used in test		Maximal quantity per 10 ml., used in test
		$mM \times 10^4$	$mM \times 10^4$		$mM \times 10^4$
L-Leucine*	+	2.2			
D-Leucine*	—		75	—	75
DL-Leucine†	+	4.4			
L-Leucylglycine‡	+	2.8			
D-Leucylglycine‡	+	128 (Ca)			
N-Methyl-DL-leucylglycine (4)§	—		50	—	100
L-Leucylglycylglycine‡	+	2.4			
D-Leucylglycylglycine‡	+	86 (Ca)			
L-Leucyl-L-tyrosine‡	+	3.2			
L-Leucinamide acetate (5)	+	30			
D-Leucinamide acetate (5)	—		53	—	158
Glycyl-L-leucine‡	+	2.5			
Glycyl-D-leucine (6)	—		106	—	103
Glycylglycyl-L-leucylglycine (7)	+	3.7			
Triglycyl-L-leucylglycine (7)	+	6.9			
Carbobenzoylglycyl-L-leucine (8)	+	1100 (Ca)			
Acetyl-L-leucine (9)	—		173	+	
Acetyl-DL-leucinamide (10)	—		58	—	262
Acetyldehydroleucine (11)	—		60	—	60
Acetyldehydroleucinamide (11)	—		58	—	116
Acetyldehydroleucylglycine (11)	—		44	—	88

\* Kindly supplied by Dr. W. H. Stein. The purity of these samples was established by the method of Moore and Stein (12).

† Merck preparation.

‡ Hoffmann-La Roche preparation.

§ The figures in parentheses are bibliographic reference numbers.

It should be borne in mind that compounds which proved to be inactive by the testing methods employed in these studies may actually be capable, under different experimental conditions, of replacing leucine as a growth factor or of sparing the leucine requirement of the leucineless mutant.

*Leucine Requirement*—Under the conditions employed in this study, the

concentration of L-leucine required to produce half maximal growth of strain 679-680 was 0.029 mg, or  $2.2 \times 10^{-4}$  mM, per 10 ml of medium (Table I). Similarly, the concentration of DL-leucine for half maximal growth was  $1.4 \times 10^{-4}$  mM. D-Leucine was inactive as a growth factor in concentrations up to  $75 \times 10^{-4}$  mM per 10 ml of medium and, in these concentrations, had neither a sparing action nor an inhibitory effect on the growth obtained in the presence of  $2 \times 10^{-4}$  mM of the L form. DL-Isoleucine, in concentrations up to  $23 \times 10^{-4}$  mM per 10 ml, was neither active in place of leucine nor inhibitory to growth in the presence of leucine.

*Utilization of Leucine Peptides*—As shown in Table I, all of the peptides of L-leucine which were tested served as growth factors for the mutant L-Leucylglycine, L-leucylglycylglycine, and glycyl-L-leucine were approximately as active as L-leucine. The activity of diglycyl-L-leucylglycine and triglycyl-L-leucylglycine was somewhat less than that of the di- and tripeptides, but even the pentapeptide had about one-third the activity of L-leucine. L-Leucinamide, however, was relatively inactive.

The suggestion was made previously (1) that peptides of an amino acid which is required for growth by a mutant strain of *Escherichia coli* must be hydrolyzed by the bacterial enzymes before the essential amino acid becomes available as a growth factor. The fact that none of the leucine peptides mentioned above was more active than leucine itself is consistent with this view. The relative activity of the leucine derivatives in promoting the growth of the *leucineless* strain is similar to that of corresponding derivatives of phenylalanine and of tyrosine for the *phenylalanineless* and *tyrosineless* strains (1). Thus, the dipeptides of the essential amino acid and glycine were approximately as active as the essential amino acid itself, while the amino acid amide was considerably less active, and the carbobenzyglycyl derivative showed only a slight activity. These differences in the rate of utilization of the leucine derivatives may be taken to indicate comparable differences in the rate of cleavage of these compounds by the bacterial peptidases.

Although the dipeptides of glycine and L-leucine were, on a molar basis, as active as L-leucine with respect to the amounts required to produce half maximal growth, these dipeptides were markedly inhibitory at high concentrations. As can be seen in Fig. 1, L-leucine was not inhibitory in concentrations up to 1 mg ( $76 \times 10^{-4}$  mM) per 10 ml of medium. Only slight inhibition was noted for L-leucylglycylglycine, diglycyl-L-leucylglycine, and triglycyl-L-leucylglycine at concentrations of 30 to  $50 \times 10^{-4}$  mM, but very striking inhibition was observed for similar concentrations of glycyl-L-leucine and L-leucylglycine. On the other hand, L-leucyl-L-tyrosine did not show significant inhibition in concentrations up to  $102 \times 10^{-4}$  mM.

microbiological assay of the resultant medium indicated the presence of L-leucine, or of a substance with L-leucine activity, in an amount equivalent to about 12 per cent of the starting material. Resting cells of the wild type strain did not bring about this conversion. The presence of some unchanged acetyl-L-leucine in the medium in which K-12 cells were grown was also demonstrated by sparing action tests with the *leucineless* mutant.

The ability of both the wild type strain and the mutant to convert acetyl-leucine to leucine, or a leucine-like compound, is in accord with the view that the metabolism of the parent strain and that of a mutant arising from it are identical, except for the metabolic reaction governed by the gene which has undergone mutation (13). In the present case, it is apparent that the mutation responsible for the leucine requirement is not connected with the metabolism of acetyl-leucine by *Escherichia coli*.

The conversion of acetyl-L-leucine to the corresponding amide appears to suppress the sparing action. As has been noted above, the conversion of an amino acid to its amide was found to reduce markedly its growth-promoting action. In the case of acetyl-L-leucine, such a conversion decreased more than 100-fold the sparing action characteristic of the parent compound.

The response of the *leucineless* mutant to acetyl-leucine is quite distinct from that of the *phenylalanineless* and *tyrosineless* mutants. For these latter mutants, acetylphenylalanine and acetyltyrosine neither filled nor spared the amino acid requirement (1). It is of interest in this connection that the original K-12 strain was found to be incapable of hydrolyzing acetyltyrosine (14).

*Effect of Derivatives of Dehydroleucine*—In order to examine further the possible metabolic rôle of dehydroamino acids (cf. (1, 14)), several derivatives of  $\alpha$ ,  $\beta$ -dehydroleucine were assayed as potential growth factors for the *leucineless* mutant. Acetyldehydroleucine, acetyldehydroleucinamide, and acetyldehydroleucylglycine were inactive in the growth tests, nor did they exert a sparing action on the leucine requirement of the mutant (Table I). In the case of acetyldehydroleucine, at least, a sparing action should have been demonstrable if the mutant was able to reduce the  $\alpha$ ,  $\beta$ -double bond to yield acetyl-L-leucine. Experiments in which the disappearance of the  $\alpha$ ,  $\beta$ -double bond in derivatives of dehydroamino acids was followed spectrophotometrically had indicated that growing cultures of *Escherichia coli* strain K-12 did not readily metabolize acetyldehydroleucine (14). The inactivity of that compound in the tests with the *leucineless* mutant strain, therefore, is not unexpected.

#### SUMMARY

The utilization of derivatives of leucine has been studied with an x-ray-induced mutant strain of *Escherichia coli* which requires for growth an

*leucineless* mutant D-leucylglycine, D-leucylglycylglycine, D-leucinamide acetate, and glycyl-D-leucine (Table I). Only the first two compounds exhibited any growth-promoting activity, which corresponded to 2 to 3 per cent of that of the L isomers. The activity of D-leucylglycine and D-leucylglycylglycine, both of which are commercial preparations, may well be due to traces of the L isomers present in the samples.

*Utilization of Acetyl-L-leucine*—Although acetyl-L-leucine did not replace L-leucine as a growth factor for the *leucineless* mutant, it did exert a sparing action on the leucine requirement. The addition of increasing amounts of acetylleucine to a culture medium containing  $2 \times 10^{-4}$  mM of L-leucine per 10 ml gave significantly more growth than was expected from the quantity of L-leucine used. This stimulation of growth is not due to

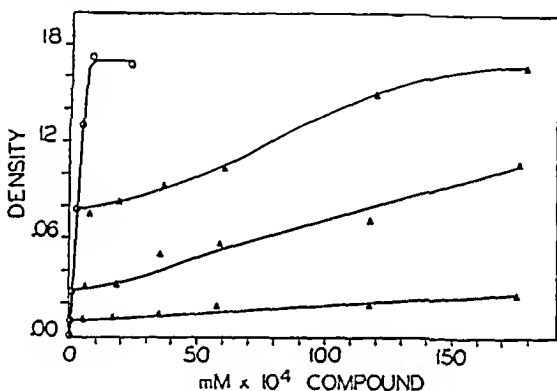


FIG 2 The sparing action of acetyl L leucine on the leucine requirement of the *leucineless* mutant of *Escherichia coli*. O L-leucine,  $\Delta$  L-leucine plus acetyl-L-leucine

traces of L-leucine admixed with the acetyl compound, since no growth-promoting action could be detected in the absence of L-leucine with concentrations of acetyl-L-leucine as high as  $173 \times 10^{-4}$  mM. The presence of even 0.3 per cent of L-leucine in such a sample of acetyl-L-leucine would have been sufficient to produce detectable growth.

As shown in Fig 2, the degree to which the requirement for L-leucine could be spared by acetyl-L-leucine was dependent upon the amount of L-leucine present in the medium. The conversion of the acetyl derivative to leucine, or to some compound with leucine activity, apparently is the result of a metabolic process associated with growth. In agreement with this view is the fact that growing cells of the wild type strain (K-12) were found to be capable of converting acetylleucine to a compound which is a growth factor for the *leucineless* mutant. When K-12 was grown for 24 hours at  $30^\circ$  in 10 ml of medium containing 2 mg of acetyl-L-leucine, a

microbiological assay of the resultant medium indicated the presence of L-leucine, or of a substance with L-leucine activity, in an amount equivalent to about 12 per cent of the starting material. Resting cells of the wild type strain did not bring about this conversion. The presence of some unchanged acetyl-L-leucine in the medium in which K-12 cells were grown was also demonstrated by sparing action tests with the *leucineless* mutant.

The ability of both the wild type strain and the mutant to convert acetyl-leucine to leucine, or a leucine-like compound, is in accord with the view that the metabolism of the parent strain and that of a mutant arising from it are identical, except for the metabolic reaction governed by the gene which has undergone mutation (13). In the present case, it is apparent that the mutation responsible for the leucine requirement is not connected with the metabolism of acetyl-leucine by *Escherichia coli*.

The conversion of acetyl-L-leucine to the corresponding amide appears to suppress the sparing action. As has been noted above, the conversion of an amino acid to its amide was found to reduce markedly its growth-promoting action. In the case of acetyl-L-leucine, such a conversion decreased more than 100-fold the sparing action characteristic of the parent compound.

The response of the *leucineless* mutant to acetyl-leucine is quite distinct from that of the *phenylalanineless* and *tyrosineless* mutants. For these latter mutants, acetylphenylalanine and acetyltyrosine neither filled nor spared the amino acid requirement (1). It is of interest in this connection that the original K-12 strain was found to be incapable of hydrolyzing acetyltyrosine (14).

*Effect of Derivatives of Dehydroleucine*—In order to examine further the possible metabolic rôle of dehydroamino acids (*cf.* (1, 14)), several derivatives of  $\alpha$ ,  $\beta$ -dehydroleucine were assayed as potential growth factors for the *leucineless* mutant. Acetyldehydroleucine, acetyldehydroleucinamide, and acetyldehydroleucylglycine were inactive in the growth tests, nor did they exert a sparing action on the leucine requirement of the mutant (Table I). In the case of acetyldehydroleucine, at least, a sparing action should have been demonstrable if the mutant was able to reduce the  $\alpha$ ,  $\beta$ -double bond to yield acetyl-L-leucine. Experiments in which the disappearance of the  $\alpha$ ,  $\beta$ -double bond in derivatives of dehydroamino acids was followed spectrophotometrically had indicated that growing cultures of *Escherichia coli* strain K-12 did not readily metabolize acetyldehydroleucine (14). The inactivity of that compound in the tests with the *leucineless* mutant strain, therefore, is not unexpected.

#### SUMMARY

The utilization of derivatives of leucine has been studied with an x-ray-induced mutant strain of *Escherichia coli* which requires for growth an



exogenous source of L-leucine All of the leucine peptides which were tested were found to serve as growth factors for the *leucineless* mutant Acetyl-leucine, however, was not an active growth factor, although it did spare the requirement of the mutant for L-leucine Derivatives of dehydroleucine were neither active growth factors nor did they spare the leucine requirement

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# A CHEMICAL METHOD FOR THE DETERMINATION OF STREPTOMYCIN IN BLOOD AND SPINAL FLUID\*

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(Received for publication, June 27, 1947)

The clinical application of streptomycin, as for any other potent drug, requires reliable information on blood levels of the drug. Microbiological assays will give estimates subject to wide variations unless a sufficient number are performed to permit statistical evaluation. The need for a reasonably simple and reproducible chemical assay for streptomycin in blood is apparent.

We have previously described chemical methods for the assay of streptomycin in clinical preparations, nutrient broth, and urine (1), which were based on the formation of maltol from streptomycin by treatment with alkali. These methods have satisfactory specificity and reproducibility, but the lower limit of sensitivity was 30  $\gamma$  of streptomycin. Since the concentration of streptomycin in the blood which is of clinical interest is in the order of magnitude of 1 to 30  $\gamma$  per ml, a more sensitive method is necessary.

The early work on the structure of streptomycin indicated the presence of a reactive carbonyl group (2) and of guanido groups (3). On acid hydrolysis (4) streptomycin is cleaved into streptidine, containing the strongly basic guanido groups, and streptobiosamine, which contains the carbonyl group. The determination of streptomycin described in this paper is based on the presence of the reactive carbonyl group together with the strongly basic groups on 1 molecule. Since the presence of these two functional groups in the same molecule is quite rare in biological materials, the method is highly specific.

In general, a substituted hydrazine can be used as a carbonyl reagent for the assay of streptomycin if the following conditions are fulfilled. The streptomycin hydrazone must be measurable by direct or indirect color formation, fluorescence, or ultraviolet absorption. The excess reagent together with the hydrazones of acidic, neutral, and weakly basic compounds must be separable from the strongly basic streptomycin hydrazone. Of particular importance in blood assays is the separation from the hydrazones of reducing sugars, amino sugars, keto acids, and from the possible cleavage products of streptomycin such as N-methyl-L-glucosamine (5) and streptobiosamine. The reagent should also be reasonably stable.

\* This paper was presented at the meeting of the Federation of American Societies for Experimental Biology, Chicago, May, 1947 (*Federation Proc.*, 6, 241 (1947)).

For example, 2,4-dinitrophenylhydrazine fulfilled these requirements, forming a hydrazone of streptomycin in acid solution at room temperature. The excess reagent and other hydrazones were separated by two extractions with butyl acetate from the acid solution, and the 2,4-dinitrophenylhydrazone of streptomycin was measured colorimetrically in alkaline solution. The lower limit of sensitivity with this reagent was 50  $\gamma$  of streptomycin, insufficient for determination in blood.

In order to measure the minute quantities of streptomycin in blood, it was expected that a fluorescent hydrazine might provide a reagent of the necessary sensitivity. Of a limited number of hydrazines of acridines, quinolines, and anthraquinones prepared and tested, 9-hydrazinoacridine hydrochloride was found to have all of the required properties, particularly the necessary sensitivity to measure 1  $\gamma$  of streptomycin.

#### EXPERIMENTAL

*Preparation of 9-Hydrazinoacridine Hydrochloride (6)*—15 gm of 9-chloroacridine, readily obtained from N-phenylanthranilic acid (7), were dissolved by heating in 400 ml of 95 per cent ethanol containing 30 ml of 85 per cent hydrazine hydrate. After refluxing for 6 hours, the hot mixture was poured into 400 ml of 6 N hydrochloric acid with vigorous stirring and external cooling. 200 gm of cracked ice were added and the mixture was refrigerated overnight. The crude product was washed three times with 1 N hydrochloric acid, five times with a mixture of ethanol and concentrated hydrochloric acid (10:1), three times with chloroform, and three times with ether saturated with concentrated hydrochloric acid. The crude material was recrystallized, half the weight of charcoal and 75 volumes of 50 per cent ethanol being used. The crystals were washed with 50 per cent ethanol, then with the ethanol-hydrochloric acid mixture until the washings remained straw-yellow, and finally with ether saturated with concentrated hydrochloric acid.

Further recrystallizations, one or two were sufficient, were carried out with 90 to 100 volumes of 50 per cent ethanol containing 1 ml of concentrated hydrochloric acid per 100 ml. After each recrystallization, the product was washed with the ethanol-hydrochloric acid mixture until the washings were straw-yellow. The final product of fine, yellow-green needles weighed 7.5 to 8.0 gm, 45 to 50 per cent of theory.

$C_{11}H_{12}N_2Cl$	Calculated	C 63.54, H 4.92, N 17.10, Cl 14.43
	Found	" 63.71, " 4.73, " 17.00, " 14.61

#### *Determination of Streptomycin in Aqueous Solution*

##### *Reagents—*

3 N hydrochloric acid

Benzyl alcohol-carbon tetrachloride mixture (10 1)

9-Hydrazinoacridine hydrochloride solution containing 5 mg of 9-hydrazinoacridine hydrochloride per 1 ml of a mixture of equal parts of water, methanol, and butanol. The reagent was stable for 1 week in the refrigerator.

*Procedure*—To 14 ml of a solution containing from 0.05 to 1.5  $\gamma$  of streptomycin per ml, was added 1 ml of 3 N hydrochloric acid followed by 3 ml of the 9-hydrazinoacridine reagent. The reaction mixture was placed in an oven at  $37^{\circ} \pm 1^{\circ}$  for an overnight period of 16 hours.

After hydrazone formation, the excess hydrazine reagent and other hydrazones which are acidic, neutral, or less basic than the hydrazone of streptomycin were separated from the basic acridylhydrazone of streptomycin by three extractions with the benzyl alcohol mixture. Carbon tetrachloride was added to the benzyl alcohol to increase the specific gravity and thus facilitate phase separation. The first extraction was with 20 ml of benzyl alcohol mixture together with 2 ml of concentrated hydrochloric acid. The benzyl alcohol and the concentrated hydrochloric acid must be added separately and not combined as a reagent. The second extraction was with 20 ml of benzyl alcohol mixture alone, and in the third extraction 2 ml of concentrated hydrochloric acid were used with the 20 ml of benzyl alcohol in order to maintain a high acid concentration. Each extraction consisted of vigorous shaking for 15 seconds. Only one set of separatory funnels was necessary, since the benzyl alcohol is the heavier, lower phase and was discarded after each extraction.

A few precautions were necessary for the successful separation by the extraction procedure. The number of samples extracted at one time should be limited to four, and the extractions should be completed as rapidly as possible in dim light to minimize the usual deleterious effect of light on fluorescence measurements. A shaded hood was used for the extractions, but the employment of red, low actinic Corning glassware may be preferred. The excess 9-hydrazinoacridine hydrochloride was removed quantitatively only if care was taken to wash traces from the stopper and mouth of the separatory funnel by rinsing with the stream of benzyl alcohol as it was added for the second and third extractions. Cello-Seal, containing only a small amount of fluorescent material, was the lubricant used for the stop-cocks.

Following the third extraction, the water phase (15.5 to 16 ml) containing the hydrazone of streptomycin hydrochloride was centrifuged, and approximately 15 ml were pipetted into a 10  $\times$  40 mm cuvette. In the Pfaltz and Bauer fluorophotometer used for measuring the intensity of the fluorescence, a Corning glass filter, No. 5113, 2 mm thick, was placed in the path of the incident beam, and a Corning filter No. 3385, 1.50 mm

in thickness, was placed between the sample and the photocell. The fluorophotometer was arbitrarily adjusted to a constant light intensity of 100 on the galvanometer scale with a standard fluorescent glass block used in the assay of riboflavin<sup>1</sup> (8). The block setting was checked before and after the reading of each unknown solution to prevent possible errors due to variation in lamp current.

*Calibration*—According to definition, 1 unit of streptomycin is equal to 1  $\gamma$  of streptomycin free base. A sample of crystalline streptomycin trihydrochloride-calcium chloride double salt (9) was the standard used in the calibration. Two micro samples were weighed, one for the solution to be used for the calibration, the other for a moisture determination in order to correct for the water in the sample (1). The procedure described was used with amounts of streptomycin varying from 1 to 20  $\gamma$ , and the fluorescence was strictly proportional to the streptomycin concentration over the entire range. The calibration should be repeated whenever a new batch of 9-hydrazinoacridine hydrochloride is obtained. In our experience the same calibration curve was obtained with each new preparation.

#### *Determination of Streptomycin in Blood*

The procedure just described was applicable to the determination of streptomycin in aqueous solution and in spinal fluid. However, when this method was applied to blood or plasma, the preparation of a protein-free filtrate was necessary prior to reaction with the hydrazine.

The deproteinizing agent must fulfil two requirements: it must not form a precipitate with the 9-hydrazinoacridine hydrochloride, and there should be minimum adsorption of streptomycin on the protein precipitate. The usual acid deproteinizing agents precipitate the 9-hydrazinoacridine unless removed prior to hydrazone formation. For example, trichloroacetic acid was suitable only if the excess trichloroacetic acid was removed from the filtrate by one extraction with benzyl alcohol.

For plasma and serum the deproteinization method of Somogyi (10) with a 1:20 dilution was found to be the simplest and did not interfere with the hydrazone formation. 1 ml of plasma containing 1 to 30  $\gamma$  of streptomycin was diluted with 17 ml of water, and 1 ml of 10 per cent zinc sulfate was added, followed by 1 ml of 0.5 N NaOH.

If the concentration of streptomycin in 1 ml of plasma was 1 to 30  $\gamma$ , a 14 ml aliquot of the filtrate was used. A higher concentration than 30  $\gamma$  of streptomycin per ml of plasma necessitated a correspondingly smaller aliquot containing not more than 20  $\gamma$  of streptomycin. This appropriate aliquot, if it was not 14 ml, was diluted to a volume of 14 ml with water, and the streptomycin was determined as previously described.

<sup>1</sup> Pfaltz and Bauer, Inc., New York, catalogue 609.

By the use of the 14 ml aliquot of the blood filtrate, seven-tenths of the total amount of streptomycin in 1 ml of plasma was measured, which was taken into account in using the calibration curve.

Some adsorption of streptomycin on the protein precipitate occurred, however, the amount adsorbed was small, since a 1:20 dilution was used in the deproteinization procedure. The recovery of 0.7 to 21.0  $\gamma$  of streptomycin added to 1 ml of normal human plasma is reported in Table I.

The average recovery was  $92 \pm 4$  per cent. Therefore, to correct for adsorption, the value obtained from the calibration curve was divided by 0.92.

TABLE I  
*Recovery of Streptomycin Added to Human Serum*

Streptomycin		Per cent recovered
Added	Found	
$\gamma$ per ml	$\gamma$ per ml	
0.7	0.7	100.0
1.4	1.2	85.0
2.1	2.0	93.4
2.8	2.7	96.5
3.5	3.1	90.0
7.0	6.6	94.0
10.5	9.4	89.3
14.0	12.9	92.2
17.5	16.5	94.5
21.0	18.8	89.5

#### DISCUSSION

The optimal conditions for hydrazone formation were studied. The reaction occurred only in acid solution, but the acid concentration was not crucial, maximal values being obtained in concentrations varying from 0.1 to 0.5 N. A concentration of hydrochloric acid near the lower limit was chosen in order to minimize possible cleavage of the streptomycin molecule.

The reaction temperature of 37° for an overnight period of 16 hours was adopted after a study of the rate of hydrazone formation at various temperatures.

From Fig. 1 it is apparent that the initial rate of hydrazone formation was rapid at 100°. However, cleavage of the streptomycin prior to hydrazone formation and the destruction of the streptomycin hydrazone prevent maximal values. Nevertheless, if a very rapid assay should be required, reaction at 100° for 25 minutes might be considered if half the sensitivity is sufficient. At 50° hydrazone formation predominated, and the de-

struction of the streptomycin hydrazone occurred slowly, but maximal values were reached at 7 to 8 hours, a technically inconvenient time. At room temperature the rate was too slow to be practical. Hydrazone formation was complete at 37° after 14 hours, and the hydrazone was stable for at least 48 hours.

The extraction with the benzyl alcohol mixture removed the excess reagent quantitatively, since the reagent blank was within 1 or 2 deflections of the instrument blank with water. The blank value for 1 ml of normal plasma or serum of various species (man, dog, cat, rat, and guinea pig) was nearly constant, and on our instrument was  $7 \pm 1$  deflections, a value within 2 to 3 deflections of the instrument blank. This average blank reading for normal plasma may be assumed with negligible error for subtraction from the total galvanometer reading when the concentration of

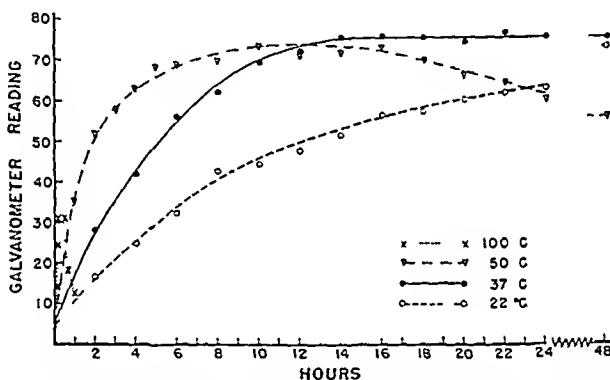


FIG 1 Rate of formation of the acridylhydrazone of streptomycin

streptomycin is greater than 5  $\gamma$  per ml of plasma. However, when maximal accuracy is necessary, particularly with samples containing low concentrations of streptomycin, a blood sample prior to streptomycin administration should be obtained and used for an accurate plasma blank.

The addition of concentrated hydrochloric acid in the first and third extractions was found to be necessary to insure a satisfactory plasma blank and to obtain optimal fluorescence. The benzyl alcohol and the concentrated hydrochloric acid should not be combined as a single reagent, since in our experience such a mixture formed measurable quantities of water-soluble fluorescent materials on prolonged standing.

The specificity of the method was studied with a series of carbonyl compounds which might interfere. Readings comparable to the reagent blank were obtained with 1 mg quantities of glucose, fructose, and galactose. Acetone, acetaldehyde, ethyl acetoacetate, and pyruvic acid did not interfere in similar amounts. Salicylaldehyde, benzaldehyde, and furfural



in quantities of 1 mg immediately formed insoluble hydrazones, but they were removed on extraction and the aqueous phase gave the usual blank reading. The hydrazones of the weakly basic cleavage products of streptomycin, streptobiosamine, and N-methyl-L-glucosamine were extracted quantitatively from the aqueous phase. Obviously, any substance which is inherently fluorescent and which is not extracted by benzyl alcohol would interfere, but such a material was not encountered in normal blood or spinal fluid.

The reproducibility was demonstrated in a series of twenty-six determinations on quantities of the standard sample varying from 0.7 to 17.5

TABLE II  
*Comparison of Chemical with Microbiological Assay in Human Serum*

Chemical assay	Microbiological assay	Microbiological per cent of chemical assay	Chemical assay	Microbiological assay	Microbiological per cent of chemical assay	Chemical assay	Microbiological assay	Microbiological per cent of chemical assay
$\gamma$ per ml	$\gamma$ per ml		$\gamma$ per ml	$\gamma$ per ml		$\gamma$ per ml	$\gamma$ per ml	
33.4	28.0	84	15.9	17.9	113	6.5	6.5	100
33.2	38.2	115	13.2	15.5	117	6.5	5.3	82
32.1	10.0	125	11.8	12.5	106	6.2	6.3	102
31.6	36.3	115	11.5	8.6	75	6.1	6.4	105
28.0	36.3	130	11.3	11.9	105	5.9	8.2	139
27.0	31.8	118	10.4	10.9	105	5.8	4.5	78
25.3	30.0	118	9.3	8.8	95	5.4	6.2	115
24.5	22.2	91	8.3	8.7	105	5.3	5.5	104
22.3	24.9	112	8.3	7.8	94	4.6	5.2	113
22.0	22.4	102	8.3	8.5	102	4.6	4.7	102
20.8	23.1	111	7.7	7.9	103	4.5	4.4	98
20.0	23.4	117	7.4	6.6	89	4.5	4.5	100
17.8	17.5	98	7.3	5.1	70	4.0	4.3	107
17.2	19.7	114	7.0	7.3	104	4.0	4.2	105
16.7	19.4	116	6.9	6.7	97	2.5	2.6	104

$\gamma$  of streptomycin. The standard deviation for the entire range was  $\pm 6.0$  per cent, for quantities of streptomycin from 3.5 to 17.5  $\gamma$ , the deviation was  $\pm 4$  per cent, and for quantities at the lower limit, 0.7 to 2.8  $\gamma$ , the deviation was  $\pm 10$  per cent.

A comparison of the chemical with the microbiological assay on forty-five samples of human serum is reported in Table II.

Over the entire range of streptomycin concentration, the microbiological value was found to be  $105 \pm 14$  per cent, if the chemical assay was arbitrarily taken as 100 per cent.

The fluorometric and the colorimetric assay, based on maltol formation,

agreed for streptomycin samples of any potency within the stated limits of error. From this observation, and the properties described by Fried and Titus (11), streptomycin B appears to behave in this test as does streptomycin. If impure preparations containing mixtures of the two streptomycins are assayed, consistent agreement with the microbiological assay can only be expected if a test organism is chosen for the microbiological assay which has identical sensitivity towards streptomycins A and B.

The distribution of the drug between plasma and erythrocytes was determined. Microbiological assays have indicated that only small amounts

TABLE III  
*Distribution of Streptomycin between Plasma and Erythrocytes of Dogs after Intramuscular Injection*

Hematocrit	Streptomycin in			
	Whole blood	Plasma	Erythrocytes (calculated)	Erythrocytes as per cent of plasma value
<i>per cent</i>	<i>γ per ml</i>	<i>γ per ml</i>	<i>γ per ml</i>	
47.0	23.5	42.5	+1.9	+4.5
52.0	17.3	32.2	+3.5	+10.8
51.0	17.0	32.8	+1.8	+5.4
48.5	14.7	28.0	+0.6	+2.2
46.5	14.2	25.8	+0.9	+3.3
44.0	13.0	24.0	-1.1	-4.8
42.0	11.0	19.0	0.0	0.0
38.0	8.5	14.0	-0.5	-3.8
49.5	7.8	14.9	+0.5	+3.4
41.0	6.5	11.0	0.0	0.0
44.0	5.0	9.0	-0.1	-1.0
49.5	3.6	8.1	-1.0	-12.5
49.5	2.5	4.8	+0.2	+3.4
Average Deviation				+0.8 ±5.7

of streptomycin, if any, entered the erythrocyte (12). The hematocrit value and the concentration of streptomycin in the whole blood and plasma were determined after injection of normal dogs with the drug.

From Table III it is apparent that no significant amounts of streptomycin were found by the chemical method in the erythrocyte. Thus under ordinary circumstances, the streptomycin concentration was measured in the plasma or serum rather than in the whole blood.

This method has been applied to the study of problems which require the accurate determination of the blood levels of streptomycin. The rate of decrease of streptomycin in the blood of man and dog following intra-

muscular injection of various amounts of the drug will be reported in a separate paper

### *Determination of Streptomycin in Spinal Fluid*

The fluorometric method has been used for the assay of streptomycin in spinal fluid. Usually deproteinization was not necessary, since the same values were obtained on any given spinal fluid with or without deproteinization. However, in certain pathological specimens of high protein content, deproteinization of 1 ml. of spinal fluid with zinc hydroxide as described for plasma might be necessary. Generally 1 ml. of spinal fluid was diluted to 11 ml. and the procedure for the determination of streptomycin in aqueous solution was followed.

TABLE IV  
*Comparison of Chemical with Microbiological Assay in Human Spinal Fluids*

Chemical assay	Microbiological assay	Microbiological assay, per cent of chemical assay
$\gamma$ per ml	$\gamma$ per ml	
20.0	13.3	66
19.1	16.1	84
17.8	15.6	88
16.3	15.6	96
15.5	10.8	70
14.7	12.1	82
14.6	14.1	97
13.4	13.4	100
9.4	7.7	82
7.8	7.5	96
5.8	4.8	83
1.5	3.3	73

When streptomycin was added to 1 ml. of normal human spinal fluid, the recovery was found to be  $100 \pm 3$  per cent. The blank with 1 ml. of normal spinal fluid was the same as for plasma, and was within 2 to 3 deflections of the instrument blank.

Comparison of the chemical with the microbiological assay on twelve samples of spinal fluid obtained from patients under streptomycin therapy is reported in Table IV.

The microbiological value was found to be  $85 \pm 11$  per cent, if the chemical assay was arbitrarily taken as 100 per cent. Agreement of the chemical with the microbiological values could not be expected in all cases, since there is a considerably larger variation in the microbiological assay of streptomycin in spinal fluid than in plasma.

*Further Applications*

The extension of this procedure to the assay of streptomycin in urine and tissues is under investigation

The principle used in this method for the determination of streptomycin is, of course, applicable to any reactive carbonyl compound which has an additional functional group or characteristic property which permits quantitative separation from the excess reagent. For example, the 9-hydrazinoacridine hydrochloride can be used for the determination of extremely small amounts of pyruvic acid or keto acids in general, if advantage is taken of the acidic group for the separation.

## SUMMARY

A fluorometric method for the determination of streptomycin in blood and spinal fluid is described. The lower limit of sensitivity is 1  $\gamma$  of streptomycin.

The basis of the method is the formation of a hydrazone of streptomycin with the fluorescent 9-hydrazinoacridine hydrochloride. The excess reagent, together with the hydrazones of acidic, neutral, and weakly basic compounds, is separated from the strongly basic streptomycin hydrazone by extraction from acid solution with benzyl alcohol. The reproducibility of the method is  $\pm 6$  per cent. The values obtained with this fluorometric method agree with the microbiological assay and the chemical assay based on the formation of maltol from streptomycin.

We wish to express our thanks to Miss Rebeckah DuBois of the Department of Medicine, Cornell University Medical College and the New York Hospital, for the microbiological assays on human serum and spinal fluid.

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# IDENTIFICATION OF SMALL AMOUNTS OF ORGANIC COMPOUNDS BY DISTRIBUTION STUDIES

## VII SEPARATION AND ESTIMATION OF NORMAL FATTY ACIDS

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*(Received for publication, March 28, 1917)*

The estimation and identification of small amounts of the lower fatty acids have always been a troublesome problem, particularly to the fermentation chemist and to the organic chemist carrying out degradative studies in which these acids are formed as fragments of a larger molecule. Several ways of approaching the problem are at hand, depending on whether or not unequivocal identification is required. Perhaps the best known preliminary approaches have been through the use of the Duclaux constants (1) and the partition method of Werkman and coworkers (2). The partition method is suitable for identifying other organic acids (3) as well but requires some supplementary procedure for demonstrating homogeneity.

More recently the possibilities in the use of partition chromatography for the identification and estimation of the lower fatty acids have been investigated (4, 5). The procedure permitted quantitative resolution under carefully standardized conditions and with a suitable silica gel. However, the position of the band for a given acid was not constant but varied with the initial amounts of acids used. This observation would suggest either interfering adsorption effects by the silica gel or a non-linear partition isotherm.

By way of comparison it was of interest, in connection with the general study under way in this laboratory, to learn whether the technique of "counter-current distribution" (6) could be satisfactorily applied to the qualitative and quantitative estimations of the lower fatty acids. This would be only a preliminary step in the extension of the study to the higher fatty acids. It was hoped that a system could be found which would not show a large shift of partition coefficient with change of concentration. Our studies have shown this latter to be possible and have also indicated that at least the lower members of the normal fatty acids can be separated readily by the procedure.

The resolution obtainable in twenty-four transfers is plainly shown by the representative distribution given in Fig. 1. This was made with a mixture of 57.1 mg. of acetic acid (b.p. 118–119°, 760 mm., m.p. 15.5–

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16°, neutralization equivalent 60.5), 51.0 mg of propionic acid (b.p. 139-140°, neutralization equivalent 73.3), 55 mg of normal butyric acid (b.p. 162-163°, neutralization equivalent 89), and 50.0 mg of *n*-valeric acid (b.p. 184-185°, neutralization equivalent 103). The system used was isopropyl ether-2.2 M phosphate buffer at a pH of 5.17 (21°). The buffer contained 1.94 moles of  $\text{KH}_2\text{PO}_4$  and 0.26 mole of  $\text{Na}_2\text{HPO}_4$  per liter.

At the completion of the distributing part of the operation it was necessary to devise some simple method for estimating quantitatively the total acid present in both phases of each tube. This was accomplished by making use of the quantitative aspects of simple successive extraction in such a manner that the complete extraction of the water-soluble acid

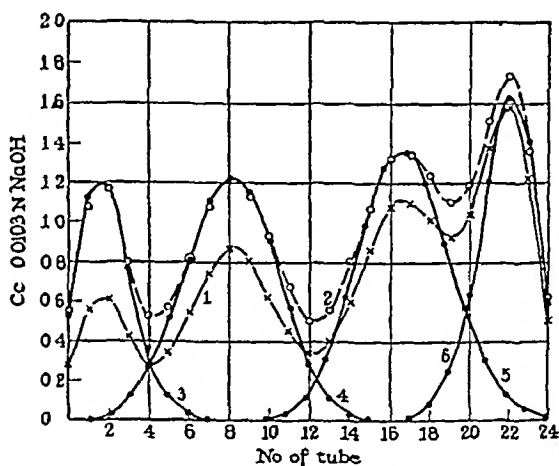


FIG. 1. Twenty-four transfer distribution of a mixture of acetic, propionic, butyric, and valeric acids. X, Curve 1, O, Curve 2, ● indicates calculated curves for separate bands, Curve 3, acetic acid, Curve 4, propionic acid, Curve 5, butyric acid, Curve 6, valeric acid.

from the aqueous phase was not required in order to calculate the total amount present per tube. In plotting a curve, however, it is not necessary to plot the total amount in each tube, since some amount proportional to the total will be just as satisfactory. This proportional amount can be represented by a single extraction or the sum of two or more extractions, as shown in Fig. 1. Two or more successive extractions permit calculation of the partition coefficient or proportionality factor by extraction equation (2).

The following procedure was employed. Each of the tubes was acidified with 1 cc of 8 M phosphoric acid, and after equilibration and centrifugation the upper layers were removed. An aliquot (1 cc) of each was titrated against standard 0.0103 M NaOH. The amount of alkali required

for each tube is shown by Curve 1. Appreciable amounts of phosphoric acid were found not to be extracted by the ether. Identical volumes of fresh isopropyl ether were then added to each of the lower layers and a second extraction made. A 1 cc aliquot of each was again titrated. The sum of this and the previous titration then gave the values shown by Curve 2. These data were then sufficient for locating the position of each band, even though complete extraction from the lower layers had not yet been achieved.

From Curve 2 the partition coefficients of 0.09, 0.50, 2.24, and 10 can be estimated (7) and theoretical curves fitted to each, as shown by Curves 3 to 6 for acetic, propionic, butyric, and valeric acids respectively. In this it is assumed that the maximum tubes of the central bands are homogeneous and that 0 and 1 of the first band and 23 and 24 of the last band are homogeneous. As can be seen from Fig. 1, the sum of these four curves approximates very closely Curve 2. The theoretical curves may therefore be used for the quantitative deductions to follow. However, the data thus far are sufficient for identification and rough estimation.

Though it can be predicted from the theory of counter-current distribution that certain of the tubes are essentially pure, since a nearly linear isotherm is indicated from the shape of the curves, it appeared of considerable interest to demonstrate this fact experimentally. Accordingly, the butyric acid remaining in Tubes 15, 16, and 17 was combined, transferred to a volume of 8 cc of isopropyl ether, and subjected to a twenty-four transfer distribution in exactly the same way and in the same system used for the original mixture. The result of this experiment is shown in Fig. 2. A theoretical curve fitted to the experimentally derived curve is also shown. The symmetry of this curve is at once evident and it will be noted that the maximum agrees very well with the maximum of the butyric acid band of the first distribution. That the acid in Tubes 15, 16, and 17 of Fig. 1 was of high purity is now unquestionable. This experiment further indicated that the deviation from a linear isotherm is small for butyric acid at the concentration range used.

The quantitative aspects of the distribution may now be developed. The leakage at the ground surface of the distribution apparatus is only a matter of a few drops with a well seated machine. Even if it should be a cc or more, the loss may be neglected in view of the total volume of 400 cc in both phases. Experimentally, any loss tends to be distributed evenly among all the tubes. A loss approximating 1 to 2 per cent, however, did occur in removing the solutions from the machine. The solutions were individually removed by means of a hypodermic syringe with a long needle and, in order to shorten the process, rinsing of each tube was neglected. For a critical experiment requiring greater precision, the tubes could be rinsed.

In calculation of the percentage of each acid in the original mixture, the first step is that of finding the total amount of acid in a given tube which is shown by the curves to be of high purity. This is the sum of that determined from the two successive extractions plus the fraction remaining unextracted, and may be calculated by formulae (1) and (2). Formula (1) permits the partition coefficient for the solvent and the acidified buffer to be calculated, where  $W_1$  is the amount from the first extraction and  $W_2$  is the amount from the second extraction.  $W_1$  and  $W_2$  may be expressed

$$(1) \quad 1 - \frac{W_2}{W_1} = \frac{Kr}{Kr + 1}$$

in terms of the volumes of the standard alkali required.  $K$  is the partition coefficient.  $r$  is the ratio of the volumes of the upper and lower phases

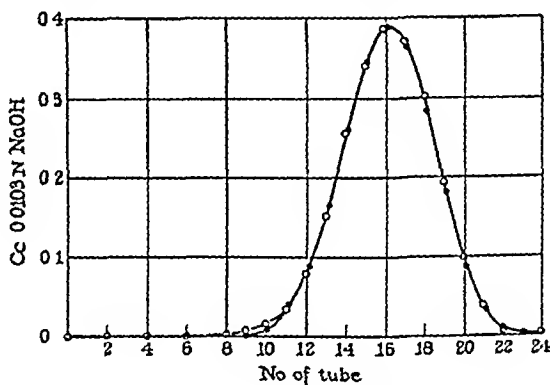


FIG 2 Redistribution of the acid in Tubes 15, 16, and 17 of Fig 1. O, experimental, ●, calculated

used in the extraction. This formula gives a sufficiently accurate estimate of  $K$  for the purpose when the substance has a linear partition isotherm and when  $W_2/W_1$  is not greater than approximately 0.7. When  $W_2/W_1$  is greater than 0.7, either the volume of the extract must be increased or a solvent used which has a more favorable partition coefficient.

From the extraction formula the fraction,  $X$ , of acid extracted on the first extraction is given by formula (2). By application of this formula

$$(2) \quad X = \frac{Kr}{Kr + 1} = 1 - \frac{W_2}{W_1}$$

to the data obtained with Tube 22 of Fig 1 the value of 0.912 is obtained for  $X$ . The total amount of valeric acid in Tube 22 (Fig 1) is therefore represented by  $1.59 \times 1.00/0.912 \times 8 = 13.96$  cc of 0.0103 N NaOH. When



A correction is made for the small amount of butyric acid shown to be present in this tube by Curve 5, the result of 13.00 cc is obtained. This is the equivalent of 13.65 mg of valeric acid.

In the binomial expansion a given term is always a fixed fraction of the total. The fraction of the total valeric acid in Tube 22 can be calculated (7) as a term of the expansion to be 0.281. The total valeric acid represented by Curve 6 is therefore  $13.65 \times 1.00/0.281 = 48.7$  mg.

Calculation of the total acid represented by Curve 5 in the same way gave 56.8 mg of butyric acid and for Curve 1, 50.3 mg of propionic acid. A sufficient fraction of acetic acid was not extracted by the two successive extractions to give the calculations in this case the desired accuracy. The amount of acid in Tube 2 was accordingly derived by two further successive extractions with 16 cc volumes of diethyl ether. This permitted 51 mg of the acid to be demonstrated in the first band under Curve 3. This value was checked by complete extraction of Tube 2.

For routine work the number of extractions and titrations made in obtaining sufficient data for interpretation could be very much reduced. It would not be necessary to extract or titrate every tube but only enough to show definitely the position of the peaks. Greater precision could be derived by extracting with 16 cc volumes instead of 8.

In order to evaluate the quantitative aspects of this distribution the homogeneity of the acid preparations used initially should be established. This is especially so in view of the fact that the method of counter-current distribution has had wide application for homogeneity studies in the war time synthetic antimalarial program and more recently in the preparation of penicillin. It has frequently shown the presence of considerable amounts of impurity when other methods have failed.

Of the four acids used in this study it appeared that the *n*-valeric acid might be most open to question. A twenty-four transfer distribution was accordingly made on 48 mg of this acid in exactly the same way as for the mixture of acids. The result obtained is shown in Fig. 3 and is again in agreement with the theory. The acid recovery calculated as above was 97 per cent. It was thought in the beginning that the most probable impurity might be isovaleric acid. Isovaleric acid in the same system was found to have a partition coefficient of 5.5, as compared to that of 7.7 for valeric acid. Appreciable amounts of the former would be evident as a deviation from the theoretical at Tubes 16 to 18.

Isobutyric acid (b.p. 153–154°, neutralization equivalent 90), on the other hand, proved to have a partition coefficient very similar to that of butyric acid and could not be satisfactorily separated in the system used. It would thus not be recognized if it were present as an impurity. It is interesting to note that Elsden (5) also was not able to separate isobutyric

acid from normal butyric acid by partition chromatography on silica gel. It was considered probable that the acetic and propionic acids used were

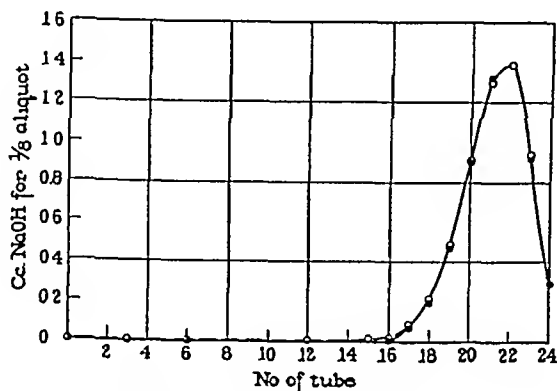


FIG 3 Twenty-four transfer distribution of valeric acid ○, experimental, ●, calculated

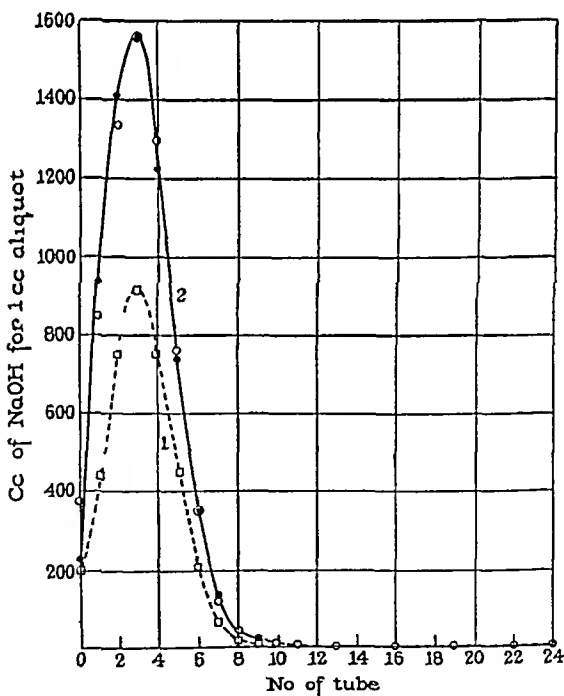


FIG 4 Twenty-four transfer distribution of acetic acid □, first extraction, ○, first plus second extraction, ●, calculated curve

of satisfactory purity for this particular study, in view of the physical constants and high recoveries obtained in Fig 1. Nevertheless, a sample

of acetic acid was distributed in the same system used for the mixture of acids, except that the volume of the upper layer was doubled. The result is shown in Fig 1. Two successive extractions of the acidified lower layers were made. They are given in Curves 1 and 2. The absence of an appreciable amount of propionic acid in the sample used is thus demonstrated.

A point of interest can be derived by comparing the distribution of valeric acid alone and the corresponding band which emerged from the acid mixture in Fig 1. The theoretical curve for the former which is in agreement with the experimental values is calculated on the basis of a partition coefficient of 7.7, and this value is in agreement with the experimentally determined partition coefficient. On the other hand, it is necessary to base the calculation of the theoretical curve for the acid on the value 10 for that obtained with the mixed acids. This is an appreciable shift of the position of the band and is a result which has been checked by a duplicate run of the four mixed acids made in a slightly different buffer. We have noted such a shift in other distributions when one of the components occupied a position either far to the left or to the right of the diagram (partition coefficient of 10 or 0.1), but have not noted such a shift as yet when the component occurred near the center of the diagram (partition coefficient of 1). In the present case it afforded a slightly better separation of butyric and valeric acids than expected. The position of the butyric acid band was essentially the same either in the mixture or alone, as the comparison of Figs 1 and 2 will show.

The agreement of the experimental curve in Fig 3 with the theoretical curve would indicate a linear partition isotherm. This has been checked experimentally over the concentration range employed.

#### SUMMARY

The use of the method of counter-current distribution for the separation and quantitative estimation of the volatile normal fatty acids ( $C_2$ - $C_5$ ) has been investigated. A system and procedure satisfactory for the purpose of estimation to within 2 or 3 per cent have been described.

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# MICRODETERMINATION OF MANGANESE IN BIOLOGICAL MATERIAL BY MEANS OF CATALYSIS\*

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*(Received for publication, June 7, 1947)*

The standard methods of manganese determination in biological material (1, 2), based on the oxidation of  $Mn^{++}$  to permanganate, are not sufficiently sensitive. Since the  $Mn^{++}$  content of animal tissues is very low, 20 to 100 gm of material are required for analysis. The color reaction of manganese dioxide and benzidine was successfully applied for the quantitative detection of  $Mn^{++}$  in tissues by Wiese and Johnson (3). This method made it possible to determine manganese from 5 gm tissue samples. However, in animal experiments it is desirable to use even less material.

It will be shown in this paper that the catalytic properties of  $Mn^{++}$  offer a possible means of microanalysis of manganese, by which mere traces of  $Mn^{++}$  can be determined from small tissue samples.

## EXPERIMENTAL

The method is based on Szebelledy's and coworkers' experimental results (4-6). It was shown by them that the oxidation of certain phenol derivatives can be catalyzed by traces of  $Mn^{++}$  ion. The most sensitive reaction for the measurement of  $Mn^{++}$  catalysis proved to be the oxidation of an aqueous solution of diethylaniline by an aqueous solution of potassium periodate. At the present time the kinetics of this reaction as well as the structure of the oxidation product are unknown. When this reaction is carried out in unbuffered solutions, no strict correlation between the rate of the reaction and the concentration of the catalyst was found. The use of a phosphate-citrate-borate buffer at pH 7.0 (7), however, increased the reproducibility of the measurements by maintaining a constant environment for the catalysis (Table I).

Markus (6) found that  $Fe^{++}$  and  $Mg^{++}$  do not alter this  $Mn^{++}$  catalysis.  $Fe^{+++}$  ion, which interferes with the catalysis in an unbuffered medium, did not show any effect in the buffered mixture, even when its concentration was 10 times as high as that calculated to be present in biological material (8). Other heavy metals did not interfere when the procedure was carried

\* Part of this work was carried out with Geza Frendl in the biological laboratory of the chemical and pharmaceutical factory of Palik and Company, Ltd., Budapest. Aided by a grant (Contract N6on-20, Task Order 11) from the United States Navy, Office of Naval Research, and by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

out at this pH and in the buffered media. The sensitivity of this catalytic reaction could be increased by using a 5 times more dilute solution of diethylaniline and potassium periodate, with the aid of the Beckman spectrophotometer<sup>1</sup>. The optimal wave-length as determined with the instrument was found to be 470 m $\mu$ .

Figs 1 and 2 were constructed from the same data, obtained with the Beckman spectrophotometer. The optical density ( $\log I_0/I_x$ ), measured at the 200th second, was plotted against the wave-length, expressed in m $\mu$  (Fig 1). A sharp maximum at 470 m $\mu$  was found.

Fig 2 shows the correlation between the amount of Mn<sup>++</sup> and the optical density measured under the same conditions as those mentioned for Fig 1. The optical density measured at the 200th second after the addition of the KIO<sub>4</sub> solution plotted against the amount of Mn<sup>++</sup> on a semilogarithmic paper gave a straight line relationship for the concentration range used.

TABLE I

*Catalytic Activity of Various Concentrations of Mn<sup>++</sup> in Phosphate Citrate Borate Buffer, pH 7.0*

The optical density of the yellow oxidation product of saturated diethylaniline by saturated KIO<sub>4</sub> was measured by means of a Lange-Roth photoelectric colorimeter, with a blue filter, at the 120th second after the addition of the KIO<sub>4</sub> solution.

	Mn <sup>++</sup> concentration micrograms per ml				
	Mn <sup>++</sup> free	0.005	0.010	0.1	0.5
	Optical density				
Mean (10 measurements)	0.116	0.124	0.134	0.169	0.198
S.D.	$\pm 0.001$	$\pm 0.0011$	$\pm 0.003$	$\pm 0.0013$	$\pm 0.0013$

This correlation made it possible to work out a method for the determination of Mn<sup>++</sup>. If one measures the catalytic activity of an unknown amount of Mn<sup>++</sup>, that is the optical density of the dye, reached at a given time (200 seconds), then the amount of Mn<sup>++</sup> can be easily read from a standard curve.

**Reagents**—1 Saturated diethylaniline solution. 4 drops of diethylaniline base are diluted with redistilled water to 100 ml. This is thoroughly shaken and is left to stand for 24 hours at room temperature in a dark place. Immediately before use the solution is filtered.

2 Saturated potassium periodate. This solution is prepared by shaking 3 gm of crystalline KIO<sub>4</sub> in 100 ml of redistilled water and treating it in

<sup>1</sup> The spectrophotometric measurements were made in the laboratory of Dr E. S. G. Barron, who kindly provided us with the use of his Beckman spectrophotometer.

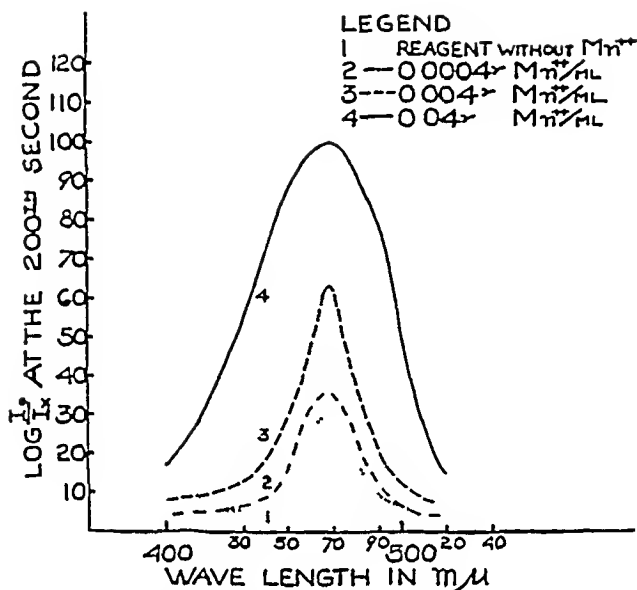


FIG 1 Correlation between the wave length used in the spectrophotometer and the optical density of the reaction mixture, containing varying amounts of  $Mn^{++}$  from 0.0004  $\gamma$  per ml to 0.04  $\gamma$  per ml. Readings were made at the 200th second after the addition of the  $KIO_4$  reagent. There is a maximum at 470  $m\mu$ .

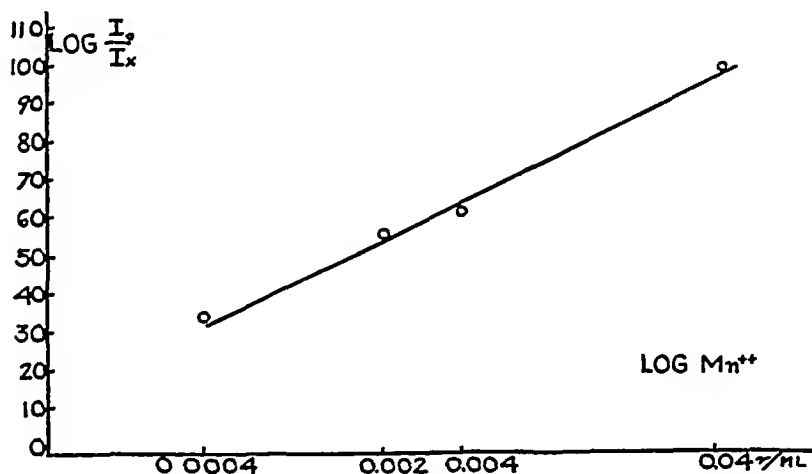


FIG 2 Relationship between the amount of  $Mn^{++}$  and the optical density of the reaction mixture measured at the 200th second after the addition of  $KIO_4$ , shown on semilogarithmic paper. The points are obtained from the same data as in Fig 1.

the same manner as Reagent 1. When a Beckman spectrophotometer is used, it is necessary to dilute Reagents 1 and 2 five times with redistilled water.

3 Manganese standard solution. Crystalline  $\text{MnSO}_4$  is dried for 3 hours at  $120^\circ$ . This substance is used for standard  $\text{Mn}^{++}$  solutions.

4 0.1  $\text{N}$  oxalic acid

5 0.1  $\text{N}$   $\text{HCl}$

6 Phosphate-citrate-borate buffer (pH 7.0)

### Procedure

The vessels used must be carefully washed with a mixture of  $\text{HCl}$  and  $\text{HNO}_3$  (1:1), and then with redistilled water five times. If the material is blood or any other fluid, it can be used as such. For each analysis (50 parallel measurements) 0.1 to 0.5 gm. of tissue or 0.5 ml. of fluid is required. The sample is measured into a platinum crucible with 10 drops of 0.1  $\text{N}$   $\text{HCl}$  and ignited at  $850^\circ$  in an electric oven for 4 hours. When the crucible is cooled, 2 ml. of 0.1  $\text{N}$  oxalic acid and 5 drops of 0.1  $\text{N}$   $\text{HCl}$  are added and the mixture kept at  $80-90^\circ$  for 20 minutes. This reduction is necessary because  $\text{Mn}^{++}$  is oxidized at  $850^\circ$  to  $\text{Mn}^{+++}$ , which is inactive as a catalyst in this case. The contents of the crucible are quantitatively washed into a 100 ml. flask and diluted to the mark with redistilled water. For each determination 2 ml. of this extract are required.

The measurements are made as follows. 2 ml. of extract plus 4 ml. of buffer (pH 7.0) are placed in a test-tube. The mixture and the reagents are kept with the standard  $\text{Mn}^{++}$  solutions at room temperature for  $\frac{1}{2}$  hour. Samples containing known amounts of  $\text{Mn}^{++}$  are prepared in the same way as tissue extracts. Immediately before the measurements are made, 2 ml. of diethylaniline solution plus 2 ml. of  $\text{KIO}_4$  reagent are added. From the moment this latter reagent is added the time must be measured exactly by a stop-watch for 200 seconds. During this time the test-tube is shaken for 30 seconds, then the mixture is poured into the photometer cup. The extinction is read at the 200th second after the addition of  $\text{KIO}_4$ . From the straight line obtained by plotting the known amounts of  $\text{Mn}^{++}$  against the  $E$  values read at the 200th second on semilogarithmic paper, the  $\text{Mn}^{++}$  concentration of the extract can be easily calculated. To insure accuracy oxalic acid and  $\text{HCl}$  are added to the  $\text{Mn}^{++}$  standard solution in the same concentration as contained in the tissue extract.

To check the described method, its results were compared with those of a known macromethod. The  $\text{Mn}^{++}$  concentration of dried brewers' yeast was first determined by the catalytic method on yeast samples of 0.1 gm. Then five parallel measurements were made with a colorimetric method (9) whereby  $\text{Mn}^{++}$  was determined as  $\text{KMnO}_4$  on 50 gm. yeast samples. With



the micromethod 1.8 to 2.0 mg of  $Mn^{++}$  per 100 gm of yeast ( $Mn^{++}$ ) were obtained, while the microdetermination yielded 2.0 to 2.1 mg per 100 gm. In ten samples  $0.04 \gamma$  of  $Mn^{++}$  was added in the form of  $MnSO_4$  to 1 gm of yeast. This amount could be recovered with a standard deviation of  $\pm 0.0018$ . Analyses of human tissues carried out by means of this method are shown in Table II, in which human muscle (quadriceps femoris), liver, and kidney were analyzed with both micro- and macromethods.

TABLE II  
*Mn<sup>++</sup> Content of Human Tissues*

Organ	Catalytic method	Macromethod (cf. (9))
	From 0.5 gm. material	From 50.0 gm. material
	<i>mg per 100 gm</i>	<i>mg per 100 gm</i>
Muscle	0.0082	0.0080
	0.0090	0.0071
	0.0083	0.0092
	0.0100	0.0084
	0.0096	0.0076
Mean	0.0090	0.0080
Liver	0.090	0.086
	0.072	0.066
	0.087	0.080
Mean	0.083	0.077
Kidney	0.030	0.025
	0.021	0.031
	0.027	0.037
Mean	0.026	0.031

#### SUMMARY

A colorimetric method, based on the catalytic properties of  $Mn^{++}$ , has been developed and used for the microdetermination of  $Mn^{++}$  in biological material.  $Mn^{++}$  catalyzes the oxidation of diethylaniline by potassium periodate to a yellow dye. The speed of this color development can be measured by means of a spectrophotometer. The speed of the reaction is a function of the amount of  $Mn^{++}$  present in the reaction mixture. With this method,  $0.0004 \gamma$  to  $0.04 \gamma$  of  $Mn^{++}$  per ml. has been determined with a precision of  $\pm 15$  per cent. 0.5 gm. of tissue or 0.1 to 0.5 ml. of biological material is sufficient for 50 parallel measurements.

The author wishes to take this opportunity to thank Dr E. M. K. Geiling for making possible the publication of this paper

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# THE ACONITE ALKALOIDS

## XX. FURTHER STUDIES WITH ATISINE AND ISOATISINE

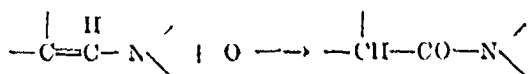
By CHARLES E. HUBNER AND WALLER A. JACOBS

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(Received for publication, June 23, 1947)

Since the alkaloids of the somewhat simpler, less toxic category of the aconite series have been dehydrogenated with selenium more successfully than aconitine and delphinine, it was of interest to see whether this advantage would extend in other directions, and further attempts with the degradation of atisine have been made. Along with other observations, certain transformations, mostly with isoatisine (1), the isomerization product of atisine, are presented here as a progress report.

On gentle oxidation with permanganate, isoatisine has been found to yield a neutral derivative, *oxoisoatisine*,  $C_{17}H_{23}O_4N$ , which contains 1 more oxygen atom than the parent substance. Its production recalls the formation of oxoaconitine and oxodelphinine from aconitine and delphinine respectively, but with the difference that, in the latter cases, the reaction consists in the oxidation to carbonyl of a methylene group adjacent to the N atom with the loss of 2 H atoms. The conversion of isoatisine,  $C_{17}H_{23}ON$ , to oxoisoatisine,  $C_{17}H_{23}O_4N$ , occurs apparently without such loss of  $H_2$  and, because of its neutral character, it must also possess a lactam structure. A possible interpretation of the reaction in this case is the oxidation to CO of an ethylenic C atom adjoining the N atom as follows:



The disappearance of a double bond during the transformation has been suggested by other observations. Isoatisine was previously shown to give a tetrahydro derivative on hydrogenation (1), whereas oxoisoatisine has now been found to yield only a *dihydrooxoisoatisine*,  $C_{17}H_{25}O_4N$ . A difference in behavior was also noted with perbenzoic acid. Oxoisoatisine reacts with only 1 equivalent of this reagent, whereas isoatisine within a short time consumes more than 1 equivalents (Table I). The reaction with isoatisine therefore goes beyond the 2 equivalents required by the 2 double bonds shown to be present by its hydrogenation to tetrahydroatisine and involves the basic function and possibly an active methylene group. Atisine and tetrahydroatisine also were found to consume 3 and 2 equivalents of the reagent respectively during the same time. With each of these bases the initial rapid consumption of perbenzoic acid was followed

by a continued more gradual reaction, until after 2 days all of the reagent added (7 to 10 equivalents) had been consumed. During such prolonged action neutral material resulted, but nothing crystalline could be isolated.

Prevost's reagent, so called silver iodobenzoate, which is known to add to an ethylenic double bond with the formation of a glycol dibenzoate (2), was likewise found to attack isoatisine. The latter with an excess of the reagent yielded a syrupy mixture of the ester. From this, on saponification, benzoic acid was split off and a crystalline *neutral substance* obtained with an apparent formulation,  $C_{22}H_{31}O_3N$ . Its neutral character showed it to be a lactam and therefore related to oxoisoatisine. If the production of the substance occurs in the conventional way, at first by formation of a glycol dibenzoate followed by hydrolysis to a glycol, further rearrangement must then occur to produce the CO group of the lactam. Its further study has been deferred for the present.

TABLE I  
*Equivalents of Perbenzoic Acid Used in Oxidations*

	0.5 hr	2 hrs	4 hrs	24 hrs	48 hrs
Oxoisoatisine	0.94			0.94	
Isoatisine	4.43	5.65	6.40	7.00*	
Tetrahydroatisine	2.15		4.50		10.00*
Atisine	3.00		3.80		8.00*
N-Ethylpiperidine	1.20	1.70	2.35	5.18	
Cyclohexene			0.99		0.99
Carvone			1.01		1.25

\* All the reagent used up

Another difference between isoatisine and oxoisoatisine was noted in their behaviors on bromination in methanol. The former in the presence of HBr was converted to a *dibromoisatisine hydrobromide*,  $C_{22}H_{33}O_2NBr_2 \cdot HBr$ , by the addition of 1 mole of bromine. Oxoisoatisine, on the other hand, yielded a neutral compound, *bromooxoisoatisine*,  $C_{22}H_{33}O_3NBr$ , by substitution with bromine. Dihydrooxoisoatisine remained unaffected on attempted bromination. The double bond which can be hydrogenated in oxoisoatisine must therefore contribute in some way to its substitution with bromine. The double bond to which bromine adds in the case of isoatisine must either disappear during its oxidation to oxoisoatisine or it has been shifted so that its reactivity has been altered. Although possible interpretations of these observations might be made, these will be left to a later occasion.

When oxoisoatisine was treated with methanol saturated with HCl at 0°,

it was converted into another *neutral substance*,  $C_{20}H_{29}O_3N$ , a formulation suggested by the analytical data. The retention of the so called N-alkyl (ethyl) group was also shown by the analysis. The apparent loss of  $C_2H_5$  during its formation from oxoisotisine may have been the result of removal from the latter of C-methyl groups on a side chain or bridge. The ultra-violet absorption spectrum of oxoisotisine (Fig. 1), like isotisine (3), showed only uneventful end absorption, but the  $C_{20}H_{29}O_3N$  product now exhibits a characteristic peak at 2950 Å (Fig. 2) which was practically unaffected when taken also in acid solution. The position and intensity

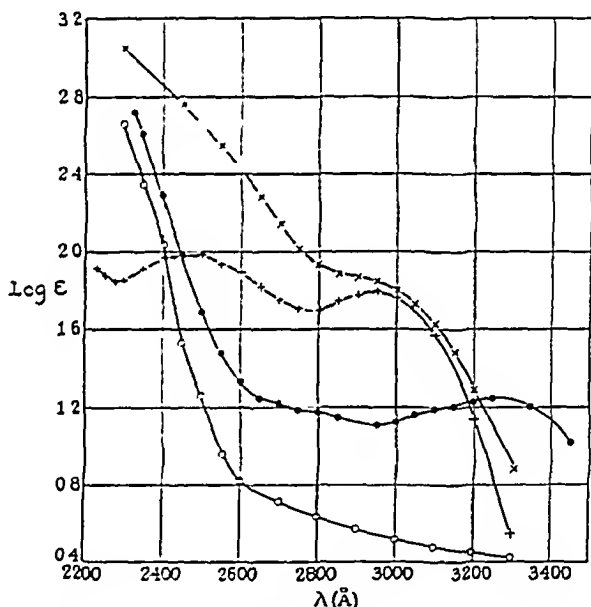
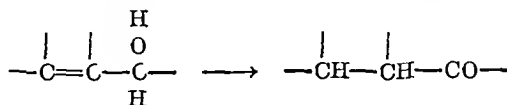


FIG. 1 ○ oxoisotisine in ethanol, ● in ethanol with excess HCl, +  $C_{20}H_{29}ON$  base in ethanol, × in ethanol with excess HCl

of the band indicate carbonyl absorption. But the presence in the compound of a CO group could not be detected directly with hydroxylamine. The formation of a carbonyl group from oxoisotisine by such treatment is not clear, but it could possibly be due to the rearrangement to CO of a newly formed, if not already present, allylic configuration as follows



This compound dissolves slowly in strong aqueous HCl but shows no basic properties when titrated with dilute acid. Solution in only strong acid may be due to a weakly basic character or to the opening of a lactam ring.

The  $C_{20}H_{29}O_3N$  derivative in a preliminary study was found to consume about 1.7 moles of  $H_2$  in 24 hours. Although a crystalline substance was obtained which differed from the starting material, no consistent analytical data were obtained with it. The hydrogenation, however, did not affect appreciably the ultraviolet absorption of the original substance, as seen from the curves given in Fig 2. A further description of the substance will be deferred until more satisfactory analytical data have been obtained.

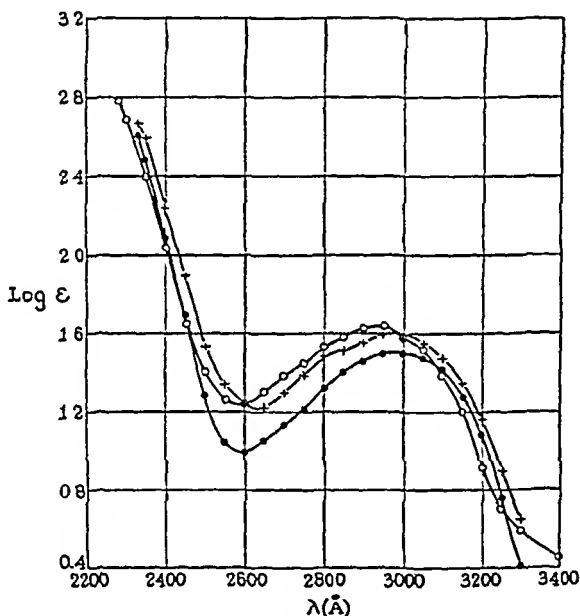


FIG 2 O  $C_{20}H_{29}O_3N$  product in ethanol, + in ethanol with excess HCl, ● hydrogenated  $C_{20}H_{29}O_3N$  product

In contrast to isoatisine, atisine is not oxidized by permanganate to a neutral product under the gentle conditions used. The reagent is consumed but slowly, and unchanged atisine is readily recovered. However, as will be presented on a later occasion, the use of a large excess of reagent under different conditions does cause oxidation. Since both atisine and isoatisine yield the same tetrahydroatisine (1), the conversion of the former into the latter must involve one or more double bonds. It appears that the double bond on the C atom adjacent to the N atom participates in the isomerization, since the resulting isoatisine has proved to be a considerably weaker base than atisine. Isoatisine is liberated completely from its salts in aqueous solution with  $Na_2CO_3$ , while atisine requires the stronger reagent,

$\text{NaOH}$  The separation of the two substances by extraction from a mixture was readily accomplished by taking advantage of this difference<sup>1</sup>

At this point mention can be made of a preliminary attempt at the high pressure dehydrogenation of atisine in benzene with Raney's nickel on  $\text{Al}_2\text{O}_3$ , made possible by the kind cooperation of Dr I A Wolff of the United States Department of Agriculture, Northern Regional Research Laboratory, at Peoria, Illinois. A base,  $\text{C}_{20}\text{H}_{23}\text{ON}$ , was obtained from the reaction mixture, m.p.  $145\text{--}150^\circ$ . This formulation was supported by the analysis of the picrate and of the hydrochloride. The interpretation of the substance will have to wait further study. The ultraviolet absorption spectrum is given in Fig. 1. The 2 carbons lost in the production of the substance may be those involved in the transformation by methanolic  $\text{HCl}$  of oxoisatisine to the  $\text{C}_{20}\text{H}_{23}\text{O}_2\text{N}$  compound.

The presence of an N-methyl group in atisine was first reported by Lawson and Topps (4), and this was accepted in our earlier studies with the substance. However, observations have been accumulating which made desirable a further study of the nature of this N-alkyl.

The occasion was taken to repeat the analytical studies also with the other aconite bases in this regard. These determinations have been made by Mr D Rigakos of this laboratory based on the method of Pregl and Lieb (5).

The results presented in Table II have consistently shown that the aconite bases, aconitine, napelline, atisine, and heteratisine, behave as if the alkyl is N-ethyl because of the solubility of the resulting ethyltrimethylammonium iodide in absolute alcohol. In the case of delphinine and staphisine the group was found to be N-methyl. But in contrast with the N-methyl determinations the N-ethyl values have been consistently low. Similar low results were obtained also with N-ethylpiperidine used as a control. Whether, in the case of the aconite bases, this is due entirely to the difficulty of the method or to the fact that the presumed N-ethyl group may occur not as such but perhaps as a bridge between the N atom and another part of the molecule remains to be determined. In addition to the N-ethyl determinations, the small N-methyl results shown in Table II may owe their origin to fractional cleavage of C-methyl groups.

As a further check on the interpretation of the N-ethyl results, isoatisine in larger amount was decomposed with the reagents as used in the N-alkyl determination. This permitted the isolation of a sufficient amount of ethyltrimethylammonium iodide and of a smaller relative amount of tetramethylammonium iodide for identification.

<sup>1</sup> It was found that the crude resinous alkaloid mixture from *Aconitum heterophyllum*, in which atisine was originally the preponderating alkaloid, after long standing (4 years) yielded almost entirely isoatisine due to isomerization. The isolation of the latter was readily accomplished by the above method.

## EXPERIMENTAL

*Oxoisatisine*—Isoatisine (0.5 gm) dissolved in 50 cc of acetone and 0.5 cc of acetic acid was gradually treated in the course of 30 minutes with 0.3 gm of potassium permanganate. After complete reduction of the reagent, the dioxide was collected with acetone. The filtrate yielded *in vacuo* a syrupy residue which was dissolved in chloroform. The latter was washed with 10 per cent HCl, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated. The residue (480 mg) crystallized when rubbed with ethanol. On recrystallization

TABLE II  
*N-Alkyl and Methoxyl Determinations*

	Found			Calculated		
	Ethyl	Methyl	OCH <sub>3</sub>	Ethyl	Methyl	OCH <sub>3</sub>
Aconitine	3.01 2.40* 2.92*	0.22	19.47 19.40 19.20	4.50		19.23
Napelline	4.78 4.12	0.22 0.39		6.60		
Heteratisine	3.60 3.48	0.32 0.23	7.95 8.11	7.42		7.93
Atisine HCl	4.53 4.53	0.38 0.21		7.65		
Delphinine	0.89	2.08	20.87		2.51	20.71
Isoatisine	4.70* 4.37*			8.46		
N-Ethylpiperidine	16.54			25.67		
N-Ethylpiperidine HCl	11.53			19.41		

\* These determinations were run as  $\text{N}(\text{CH}_3)$  but calculated for  $\text{N}(\text{C}_2\text{H}_5)$

from ethanol-water, 110 mg of oxoisatisine were obtained, m p, 250–260° after partial softening at 215°

$\text{C}_{13}\text{H}_{13}\text{O}_3\text{N}$  Calculated, C 73.48, H 9.26, found, C 73.37, H 9.14

The rather ill defined melting point was not improved by attempted purification. Sublimation at 0.05 mm and 200° yielded a crystalline substance, m p 250–255°, with partial melting at 225°

$[\alpha]_D^{20} = -39^\circ$  ( $c = 0.91$  in chloroform)

Found, C 73.25, H 9.11

This sublimate after recrystallization from ethyl acetate melted at 240–247° with softening at 230°

Found, C 73.47, H 9.20



Another preparation of oxoisoatisine after one recrystallization from ethyl acetate melted at 235–240°

$$[\alpha]_D^{25} = -37^\circ \text{ (c = 0.91 in chloroform)}$$

Found, C 73.31, H 9.08

*Bromooxoisoatisine*.—A freshly prepared 0.0977 M solution of bromine in methanol was added dropwise to 76.2 mg of oxoisoatisine in 3 cc of methanol. The addition was attended by immediate absorption of bromine. After 2.20 cc had been added, a definite excess of reagent remained. The theory for 1 equivalent is 2.17 cc. An additional 0.3 cc of reagent was added and, after a half hour, an excess persisted. The methanol solution was concentrated and the residue was dissolved in chloroform and washed with a sodium bisulfite solution. After drying and concentration, the residue obtained was recrystallized twice from methanol-water. The yield was 30 mg, and the melting point 172–175°. The substance formed rhombs.

$$[\alpha]_D^{25} = -73^\circ \text{ (c = 0.76 in ethanol)}$$

C<sub>12</sub>H<sub>11</sub>O<sub>3</sub>NBr    Calculated, C 60.25, H 7.38, found, C 60.27, H 7.25

*Dibromoxoisoatisine Hydrobromide*.—Isoatisine (305 mg) dissolved in 10 cc of methanol was titrated with freshly prepared 0.245 M bromine in methanol, during which 3.5 cc were rapidly decolorized (1 mole = 3.6 cc). A further 0.5 cc was added and then, carefully, 48 per cent HBr until acid to Congo red. Excess bromine persisted for several hours. After standing in the refrigerator 80 mg of colorless needles were collected. The filtrate on concentration yielded a second crop. The combined material was recrystallized from methanol and gave 120 mg, m.p. 212–215° with decomposition.

$$\text{C}_{12}\text{H}_{11}\text{O}_3\text{NBr}_2 \cdot \text{HBr} \quad \text{Calculated, C 45.20, H 5.87, found, C 45.12, H 5.98}$$

The hydrobromide is difficultly soluble in water. The base was liberated with alkali and extracted with benzene, but could not be obtained in crystalline form.

*Dihydrooxoisoatisine*.—84 mg of oxoisoatisine dissolved in 5 cc of methanol were hydrogenated with 41 mg of platinum oxide catalyst and absorbed 1.3 equivalents of H<sub>2</sub> within  $\frac{1}{2}$  hour. At the end of 3 hours, no further absorption was noted. The methanol filtrate was concentrated to dryness and the residue was recrystallized twice from ethyl acetate. 65 mg of stout needles were obtained, which melted at 219–223°.

$$[\alpha]_D^{25} = -38^\circ \text{ (c = 1.21 in chloroform)}$$

C<sub>12</sub>H<sub>13</sub>O<sub>3</sub>N    Calculated, C 73.07, H 9.77, found, C 73.15, H 9.72

On attempting to brominate dihydrooxoisatisine as described above, no apparent absorption of halogen occurred. Even after standing with an excess of bromine for 2 hours, the starting material was recovered. The melting point of the latter was 215–222° and of a mixture with the original substance 219–223°.

Found, C 73.15, H 9.52

*Prevost's Reagent on Isoatisine, Substance  $C_{22}H_{31}O_3N$* —100 mg of isoatisine were shaken with 750 mg (4 equivalents) of Prevost's reagent in 30 cc of dry benzene for 2 days. An excess of reagent persisted. After filtration of the silver salts, a negligible amount of basic substance was extracted with 10 per cent HCl. The benzene phase, when dried and concentrated, yielded a neutral syrup (180 mg) which could not be crystallized. 0.16 gm was saponified for 1½ hours in 15 cc of 0.15 N NaOH and 10 cc of methanol. The solution was neutralized with dilute acid and concentrated to small volume. After addition of excess HCl, the suspension was repeatedly extracted with benzene. A sodium bicarbonate extract of the benzene extract yielded 20 mg of benzoic acid, m p 120°. The remaining neutral fraction after evaporation of the benzene crystallized from ethanol and gave 20 mg of long needles, m p 225–235°. A mixture of this substance with oxoisatisine melted at 200–210°.

$C_{22}H_{31}O_3N$  Calculated, C 73.89, H 8.76, found, C 73.78, H 8.58

*Perbenzoic Acid Oxidations*—For these determinations 0.2 to 0.3 mm of the test substance was dissolved in 1 cc of chloroform and the solution was made up to 10 cc with 0.2 M perbenzoic acid in chloroform. 2 cc aliquots were removed at intervals for iodometric determination of the reagent excess. Blanks were run with the reagent alone to correct for slow decomposition at room temperature. As controls, cyclohexene, carvone, and N-ethylpiperidine were examined. The results are recorded in Table I.

*Oxoisatisine and HCl in Methanol*—To 5 cc of methanol saturated with dry HCl at 0° were added 65 mg of oxoisatisine. After 7 days in a sealed tube at room temperature, the mixture was concentrated to dryness *in vacuo* and redissolved in chloroform. When shaken with water, the material was extracted into the aqueous phase. The latter was made alkaline and reextracted with benzene. The latter yielded, when dried and concentrated, 75 mg of a syrup which crystallized from dilute ethanol. After two recrystallizations from this solvent, 30 mg of stout needles were obtained, which melted at 197–202°. The substance was halogen-free and contained no methoxyl.

$$[\alpha]_D^{25} = -17^\circ \text{ (c = 0.91 in chloroform)}$$

$C_{10}H_{10}ON$	Calculated	C 72.15, H 8.82, (N) $C_2H_5$	8.76
	Found	" 72.12, " 8.98, "	8.80

A second preparation melted at 196–198°

Found, C 72.60, H 8.99

This substance dissolves very slowly in 10 per cent HCl, from which it can be recovered with alkali. In methanol solution it consumed no HCl against alizarin red S as indicator. Isotisine can be quantitatively titrated against this indicator. No crystalline material could be obtained when atisine or isotisine was similarly treated with methanol saturated with HCl.

*Catalytic Dehydrogenation of Aisine*—5 gm of atisine hydrochloride which had been decomposed with NaOH and extracted as the base with benzene were heated in benzene solution at 325° with 3 gm of Raney's nickel catalyst on alumina (G) for 10 hours. The benzene filtrate was then extracted with 10 per cent HCl and followed by 5 per cent NaOH. 1.5 gm of a neutral fraction were recovered from the benzene phase. NaOH was added to the acid solution and on extraction with benzene 2.5 gm of a basic fraction were recovered. The amount of acidic substances proved negligible. The neutral fraction was chromatographed through  $Al_2O_3$ , but none of the subfractions was sufficiently promising for further study. The basic fraction was also chromatographed on  $Al_2O_3$ , and with benzene a lower yellow and upper brown band developed. The lower band was eluted with benzene and yielded 0.80 gm of a yellow, rather mobile oil. The upper band was removed with 30 per cent methanol in benzene and gave 1.15 gm of material. The latter was a dark brown tarry substance and yielded nothing crystalline. The first fraction in ethanol was treated with an equal weight of picric acid in ethanol. 1 gm of yellow needles of the picrate was obtained, which melted at 254–256°.

$C_{20}H_{22}ON \cdot C_6H_3O_7N_3$	Calculated	C 59.07, H 6.11, N 10.60
	Found	" 59.16, " 5.98, " 10.56

When the picrate was decomposed with aqueous NaOH and extracted with benzene, 0.6 gm of the oily base was recovered. A portion of the latter was converted to the hydrochloride and recrystallized from ethanol-ether. It formed fine needles, m p 230–235°.

$$[\alpha]_D^{25} = +31^\circ \text{ (c = 1.27 in } H_2O \text{)}$$

$C_{20}H_{20}ON \cdot HCl$	Calculated, C 71.49, H 9.01, found, C 71.32, H 9.21
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The salt dissolved in a small volume of water gave with ammonia the crystalline base. After recrystallization from ethanol-water, the fine needles obtained melted at 145–150°.

$C_{20}H_{29}ON$	Calculated	C 80.20, H 9.77, (N) $C_2H_5$ 9.68
	Found	" 79.98, " 9.89, " 2.49

*Methyl and Ethyl Iodides from Isoatisine*—300 mg of isoatisine, 200 mg of phenol, 1 gm of ammonium iodide, 0.5 cc of acetic anhydride, and 1.5 cc of hydriodic acid were placed in the conventional micro N-alkyl apparatus. A 3 per cent solution of trimethylamine in 75 per cent ethanol was used as the absorbent in the receivers. After carrying out the decomposition according to the analytical procedure (5), the contents of the receivers were evaporated to dryness *in vacuo*. When the residue was digested with 30 cc of absolute ethanol, 35 mg of insoluble material remained. After recrystallization from methanol-water this fraction yielded 10 mg of tetramethylammonium iodide.

$C_4H_{12}NI$	Calculated, C 23.88, H 5.97, found, C 23.77, H 5.79
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From the ethanol-soluble fraction 55 mg were recovered. This was dissolved in 25 cc of acetone and slowly concentrated. Three successive crops of crystals of 5 mg each were discarded. From a volume of 5 cc, 30 mg of the long needles of ethyltrimethylammonium iodide were collected.

$C_5H_{11}NI$	Calculated, C 27.91, H 6.56, found, C 27.62, H 6.44
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A control determination with 200 mg of benzylethylamine gave 38 mg of ethyltrimethylammonium iodide. A blank run with the reagents proved negative.

*Isolation of Alkaloids from Old Crude Aconitum heterophyllum Extract*—20 gm of the light brown resin of total crude alkaloid fraction from *Aconitum heterophyllum* which had been extracted 4 years previously were dissolved in 200 cc of benzene. The solution was extracted twice with 50 cc of 10 per cent  $H_2SO_4$ . The benzene phase yielded 1 gm of a neutral resin. With vigorous stirring, 12 cc of 20 per cent  $Na_2CO_3$  followed by 20 cc of 4 per cent  $Na_2CO_3$  were added dropwise. A small amount (2 gm) of dark brown gum which separated was recovered with ether but yielded nothing crystalline. A further 25 cc of 20 per cent  $Na_2CO_3$  were carefully added with stirring to the aqueous phase. From the mixture, which had become faintly alkaline to phenolphthalein, a copious yellow gum separated and was extracted with benzene. 13.5 gm of resin obtained from this extract were dissolved in a minimum of hot ethanol and diluted with water to turbidity. After cooling, 8 gm of isoatisine which crystallized were collected, m p  $149-151^\circ$ ,  $[\alpha]_D^{25} = -15.5^\circ$  ( $c = 0.98$  in toluene). The reported constants (1) are, m p  $150-151^\circ$  and  $[\alpha]_D^{25} = -16.5^\circ$  ( $C = 1.15$  in toluene).

$C_7H_9O_2N$  Calculated, C 76.91, H 9.69, found, C 77.27, H 9.36

From the alcoholic mother liquors on concentration 1 gm. of material was obtained which melted from 130–200°. After two recrystallizations from ethyl alcohol 0.1 gm. of pure heteratisine resulted, m.p., 269–271°. No depression was noted when mixed with an authentic sample.

$C_7H_9O_2N$  Calculated, C 67.47, H 8.50, found, C 67.71, H 8.40

The aqueous phase after extraction of the isoatisine was made strongly alkaline by the addition of 20 gm. of KOH. By repeated extraction with benzene 2 gm. of a resinous syrup were obtained which could not be crystallized directly but crystallized as the hydrochloride. The yield of atisine hydrochloride was 0.35 gm., m.p. 300–310°,  $[\alpha]_D^{25} = +27.6^\circ$  ( $c = 2.0$  in  $H_2O$ ). The recorded constants (1) are as follows: m.p. 311–312°,  $[\alpha]_D^{25} = +28^\circ$  ( $c = 1.0$  in  $H_2O$ ).

$C_7H_9O_2N \cdot HCl$  Calculated C 69.52, H 9.02, found, C 69.56, H 9.22

All analyses were performed by M. D. Rigakos.

#### SUMMARY

Isoatisine,  $C_{22}H_{33}O_2N$ , is oxidized with permanganate under gentle conditions to a neutral lactam, oxoisoatisine,  $C_{22}H_{33}O_3N$ . The transformation must involve the loss of an ethylenic double bond on a C atom adjacent to the N, as shown by hydrogenation studies and by the change in behavior with bromine. This double bond must also participate in the isomerization of the strong base atisine to the weaker base isoatisine.

Other transformation products are described which were obtained by the action of silver iodobenzoate on isoatisine, of HCl in methanol on oxoisoatisine, and by the high pressure catalytic dehydrogenation of atisine.

The group which reacts as N-alkyl in the aconite bases aconitine, napelline, atisine, and heteratisine has been shown to be N-ethyl, whereas in delphinine and staphisine it is N-methyl.

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# STUDIES ON THE NATURALLY OCCURRING PENICILLINS AN ASSAY METHOD FOR PENICILLIN G

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(Received for publication, December 27, 1946)

The present trend toward high purity in commercial penicillin, with emphasis on the penicillin G, has increased the need for more accurate methods of analysis. Since crude penicillin is known to be a mixture of two or more of the naturally occurring penicillins (1) and since biochemical methods of assay become complicated and uncertain when more than two penicillins are present in a mixture, chemical and physical methods seemed to offer the best hope for success.

The first step in determining the percentage of penicillin G in a product is to ascertain the total amount of all the penicillins present. Several chemical methods for the assay of total penicillin have been proposed. Herriott (2) described a method based on heating penicillin in acetate buffer, pH 4.6, and determining the increase in ultraviolet absorption at 322 m $\mu$  with a spectrophotometer. Scudi (3) has reported a colorimetric method based on the reaction between a special dye and penicillin, which is followed by removal of the unused dye and measurement of the combined dye. The titrimetric procedures include the iodometric method of Alicino (4) and the penicillinase method of Murtaugh and Levy (5). Two titrimetric methods have been developed in this Laboratory.<sup>1</sup> One is dependent on alkali inactivation in conjunction with hydrogen peroxide and is similar in principle to the penicillinase method but more rapidly accomplished. The other method involves only inactivation with alkali, followed by back titration of the excess alkali.

The method which we propose for the determination of total penicillin involves merely reading the optical rotation of a solution of known concentration. The specific rotation of pure sodium penicillin G is  $+298^{\circ} \pm 3^{\circ}$ , whence the molecular rotation is  $106^{\circ} \pm 1^{\circ} \times 10^3$ . This value appears, within the limits of experimental error, to be the same for the other varieties of penicillin, and since these differ relatively little in molecular weight the specific rotation of a sample may be taken as a measure of the total penicillin content. Some decomposition products of penicillin possess optical activity, but observations made on a large series of samples

<sup>1</sup> Chas. Pfizer and Company report to the Committee on Medical Research, Office of Scientific Research and Development, August 13, 1945.

have shown this activity to be insignificant. In fact, almost all commercial penicillin, 800 units per mg or over, has given extremely accurate values by this method, as shown in Table I. In cases of doubt, total penicillin may be checked by biological means (6) or by one of the chemical methods previously mentioned.

Our procedure for the estimation of penicillin G is based on the fact that the absorption spectrum of sodium penicillin G in the region 250 to 280  $m\mu$  contains the characteristic "breaks" of the benzyl group at 258 and 263  $m\mu$  (Fig 1, Curve 3). In contrast, other penicillins not containing an aromatic side chain give a non-characteristic absorption with very low values for the optical density in the range 260 to 280  $m\mu$ . Penicillin X<sup>2</sup> has a very high

TABLE I  
*Comparison of Polariscopic and Iodometric Assays for Total Penicillin Content*

Sample No	Polariscopic	Iodometric
	<i>per cent</i>	<i>per cent</i>
1	100	100
2	98	99
3	97	96
4	96	94
5	96	95
6	97	96
7	96	95
8	95	92

optical density at 280  $m\mu$ , whereas the other species have a minimal or low absorption at this wave-length. Macpherson's modification of the Pauly reaction (7) was adapted to the estimation of penicillin X in the presence of a large amount of penicillin G. Sodium penicillin G gives a definite yellow color with the Pauly test, but the presence of more than 0.5 per cent of penicillin X is indicated by the development of a pink color. As can be seen by a consideration of the absorption spectra, the presence of penicillin X will greatly affect the accuracy of the penicillin G determination, and the method reported here is not recommended for samples containing more than 1 per cent penicillin X. Fig 2 shows the absorption spectrum for essentially pure sodium penicillin X (Curve 1), while Fig 1 shows the absorption for a mixture of 95 per cent penicillin G with 5 per cent penicillin X (Curve 1).

The assay method for penicillin G consists of measuring the optical

We wish to thank Dr Henry Welch of the Food and Drug Administration for a supply of sodium penicillin X.



density at  $263\text{ m}\mu$  and subtracting from this figure the optical density at  $280\text{ m}\mu$ . This difference in optical density is then representative of the amount of penicillin G present. A graph showing the optical density difference plotted against per cent penicillin G in the sample is shown in

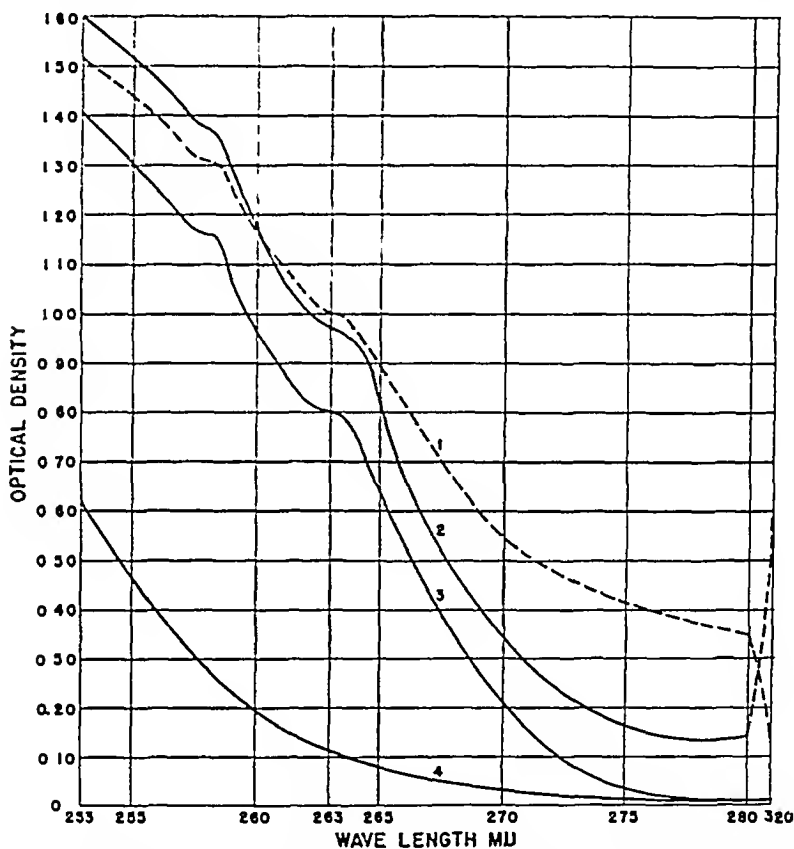


FIG 1 Ultraviolet absorption spectra of crystalline sodium penicillins in 1 cm cells (1.8 mg per ml). Curve 1, 95 per cent penicillin G plus 5 per cent penicillin X, Curve 2, commercial penicillin G (1600 units per mg), Curve 3, pure penicillin G (1667 units per mg), Curve 4, penicillin K, also identical curve for a mixture of penicillin F species.

Fig 3 The instrument which we have used is a Beckman quartz spectrophotometer.

The presence of small amounts of penicillin decomposition products causes a shift in the absorption spectrum which results in higher optical densities. When this decomposition is sufficient to give an optical density higher than 0.10 at  $280\text{ m}\mu$ , purification of the sample is necessary and can

be accomplished by the conversion of the penicillins to the ammonium salts

When the optical density at 280  $m\mu$  is 0.10 or less, spectrophotometric readings can be made directly in the manner used below for the purified ammonium salt. However, this condition is comparatively rare and for

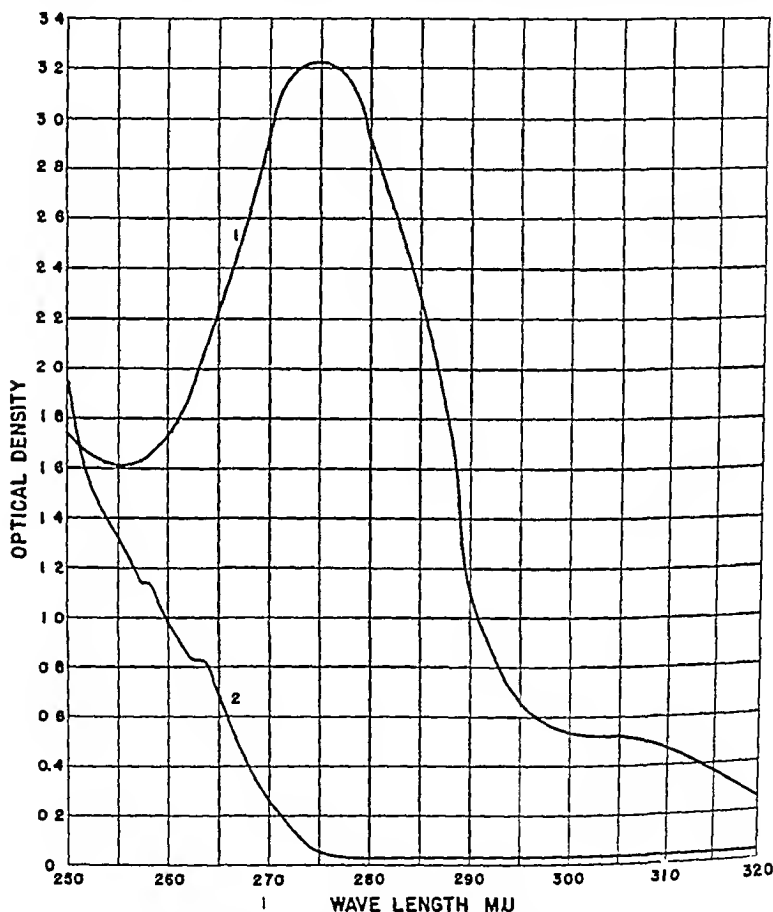


FIG 2 Ultraviolet absorption spectra of crystalline sodium penicillins in a 1 cm cell (1.8 mg per ml) Curve 1, penicillin X, Curve 2, penicillin G

the most part a purification will be necessary. Two procedures are used. Procedure A is useful with crystalline penicillin salts or yellow amorphous penicillins having a potency of 1400 units per mg or better. Procedure B is necessary with some crude amorphous products. The need for a separate method for the more crude samples of penicillin will become obvious from a study of the yield of ammonium salts obtainable from different qualities of

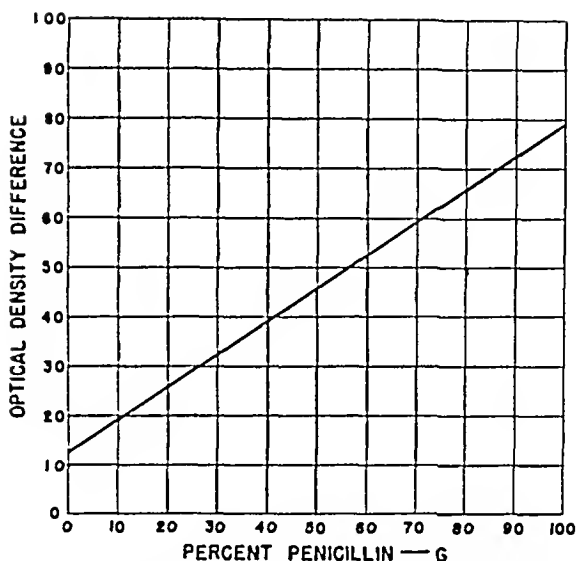


FIG 3 Change in optical density difference (263 to 280  $m\mu$ ) with increase in per cent penicillin G at a concentration of 1.8 mg per ml of penicillin

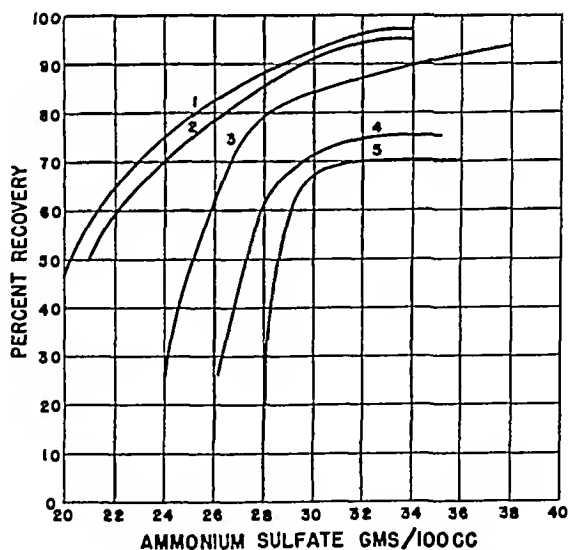


FIG 4 Recovery of ammonium penicillin with increasing ammonium sulfate concentration. Curve 1, crystalline sodium penicillin G (1667 units per mg), Curve 2, amorphous sodium penicillin (1400 units per mg), Curve 3, same (1250 units per mg), Curve 4, same (1000 units per mg), Curve 5, same (900 units per mg). Penicillin concentration, 1.8 per cent, pH 7,  $-5^{\circ}$  for 1 hour

product (Fig 4) In either case, the total penicillin must be determined as indicated previously

### EXPERIMENTAL

*Procedure A*—A 180 mg sample of the product to be analyzed for total penicillin was dissolved in 10 ml of freshly boiled and cooled distilled water at 22–24° The angular rotation was determined within a period of 10 minutes and the specific rotation calculated in the usual manner

A solution of pure penicillin G, similarly prepared, is used as a standard in the polariscopic assay Subsequent determinations of penicillin concentrates are based on this standard Pure sodium penicillin G, as determined in our laboratory on numerous samples, has a specific rotation of  $+298^{\circ} \pm 3^{\circ}$  at 25° The per cent total penicillin was calculated as follows

$$\frac{\text{Specific rotation of sample} \times 100}{\text{Specific rotation of pure sodium penicillin G}} = \% \text{ total penicillin}$$

Results obtained with samples of penicillin from a number of manufacturers are shown in Table I

To each 5 ml of a solution from the polariscopic assay were added 18 gm of pure ammonium sulfate and 1 drop of 3 per cent ammonium hydroxide The mixture was slowly cooled to  $-5^{\circ}$  with stirring, and held at this temperature 1 hour The crystalline slurry was filtered through a pre-chilled semimicro Buchner funnel and the filter cake was washed with 1 to 3 ml of ice-cold 40 per cent ammonium sulfate solution The precipitate was dissolved in 5 ml of water and the penicillin determined in both this solution and the mother liquor polariscopically

On the basis of the polariscopic assay, the purified ammonium salt was diluted to a concentration equivalent to 1.8 mg of total sodium penicillin per ml The solution was placed in a 1 cm quartz cell and the optical density was determined for the wave-lengths 263 and 280  $m\mu$  From these values, the optical density difference was calculated

$$\begin{aligned} &(\text{Optical density at } 263 \text{ } m\mu) - (\text{optical density at } \\ &280 \text{ } m\mu) = \text{optical density difference} \end{aligned}$$

With this value, the per cent of sodium penicillin G in the sample may be determined from Fig 3 Pure sodium penicillin G at a concentration of 1.8 mg per ml exhibits an optical density difference (263 to 280  $m\mu$ ) of 0.79

*Notes*—By the ammonium salt precipitation method, materials of 1400 units per mg quality are sufficiently purified to give absorption spectra approaching those of the pure penicillins (Fig 1) In the case of the precipitated ammonium salt, more latitude is permissible in regard to the optical density at 280  $m\mu$  and this may be considerably higher than the

0.10 hour on the sodium salt without interfering with the accuracy of the determination. As will be seen from Fig. 1, the yield of ammonium salt from material of 1100 units per mg is better than 90 per cent. It has been found that the proportion of the various penicillins in the mother liquor is essentially the same as in the precipitate and no correction is necessary for this difference from a quantitative yield. This is illustrated by two examples chosen from a large number of experiments.

To 10 ml. of a solution containing 120 mg. of sodium penicillin G, 10 mg. of penicillin K, and 10 mg. of penicillin F was added sufficient ammonium sulfate to give a concentration of 36 gm. per 100 ml. On standing at 10–15° for 2 hours, a 95 per cent recovery of the penicillins, as the ammonium salts, was obtained by filtering and washing with cold saturated ammonium sulfate. The polariscopic-ultraviolet absorption assay showed 83 per cent of penicillin G and 17 per cent of other penicillins. The amount of penicillin G as added to the original sample was 85.7 per cent.

Another sample was prepared from 210 mg. of sodium penicillin G and 60 mg. of sodium penicillin F. It was treated with 20 ml. of saturated ammonium sulfate and 95 per cent of the penicillins were recovered. The polariscopic-ultraviolet absorption assay showed 80 per cent of penicillin G, which exactly corresponds with the amount introduced.

Fig. 1 illustrates the absorption spectra of various samples. Curve 4 was obtained by plotting the values for sodium penicillin K and a mixture of F species penicillins in which the penicillin G content was essentially zero. These two penicillin species gave identical curves. Curve 3 represents crystalline sodium penicillin G of practically 100 per cent purity as regards both total penicillin and penicillin G. The minimal value at 280  $m\mu$  and the low peak at 320  $m\mu$  indicate the absence of penicillin degradation products. Curve 2 represents a crystalline sodium penicillin G in which decomposition has occurred, and the high optical density values at 280 and 320  $m\mu$  attest this.

The per cent sodium penicillin G, as mentioned in this paper, refers to a ratio of the amount of this species divided by the amount of total penicillin present. The value thus obtained, when considered in comparison with the per cent of total penicillin present, as determined by the polariscopic assay, may be used to find the per cent of penicillin G in the sample.

*Procedure B*—The sample was prepared for polariscopic assay in exactly the same manner as in Procedure A. A somewhat larger sample was used in this case because two different ammonium sulfate precipitations were performed. Total penicillin was determined as in Procedure A and one-half of the solution was converted to ammonium salt exactly as previously outlined. The other half of the solution was treated with 2.1 gm. of pure ammonium sulfate and 1 drop of 3 per cent ammonium hydroxide for each

5 ml This mixture was cooled to  $-5^{\circ}$  with stirring and held at this temperature for 1 hour The ammonium penicillin was filtered and washed as before and then total penicillin on both precipitate and mother liquor was determined The two purified ammonium salt solutions were diluted to 1.8 mg per ml on the basis of the polariscopic assay and the difference in optical density determined as described under Procedure A Consideration of the different values for the percentage of penicillin G in the total penicillin ammonium salts compared with the yield from the crude penicillin will enable one to extrapolate with reasonable accuracy to give the correct per cent of sodium penicillin G in the sample

*Notes*—The method of extrapolation gives results of sufficient accuracy for the more crude penicillins For example, penicillin of 1000 units per mg, shown in Fig 4 (Curve 4), gave a 62 per cent yield of total ammonium penicillins, of which 88 per cent was penicillin G, when the concentration of ammonium sulfate was 30 per cent (1.8 gm added to 5 ml) When this same sample was precipitated at 34 per cent ammonium sulfate concentration (2.1 gm added to 5 ml), the penicillin G content was determined as 90 per cent and the yield of penicillin was 74 per cent Then the per cent of penicillin G was plotted against the per cent yield for ammonium penicillin From experience with samples of somewhat higher potency, from which several points on the curve have been obtained, this was assumed to be a straight line function Therefore, on drawing a straight line through the two points at 88 and 90 per cent, we find the value of 94 per cent for the theoretical 100 per cent recovery

#### DISCUSSION

The method as outlined in this report has been for the determination of sodium penicillin G in sodium penicillin mixtures It is obvious that by using other salts as standards, the method may be applied to any salts of penicillin wherein the base radical has no absorption which interferes in the 260 to 280  $m\mu$  range Moreover, other wave-lengths and other concentrations of ammonium sulfate may be used if desired Variations will be apparent to any worker who applies the method to his particular problem, and further information about the sample may be gained by determining values at additional points The method reported has been used routinely for several months in this laboratory and is felt to be extremely accurate on high quality material, even with amorphous penicillin having a potency of 900 units per mg the accuracy is about  $\pm 3$  per cent

#### SUMMARY

A polariscopic-ultraviolet absorption method, including an ammonium salt precipitation, is described by which penicillin G may be determined in

salts of mixed penicillins This method is recommended for white crystalline commercial products and for yellow amorphous penicillin of potency as low as 900 units per mg

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# THE PURIFICATION OF HISTAMINE FOR BIOASSAY\*

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(Received for publication, June 17, 1947)

The purification of histamine for assay purposes has been the subject of reports by several investigators (1-9). The available methods are not readily applicable to large numbers of samples, especially when there is less than 2  $\gamma$  of histamine per sample. The ultrafiltration method of Emmelin probably is the least objectionable from the standpoint of time consumed and equipment required, but this method has yet to be tested thoroughly, and one should suspect that its selectivity for histamine may be inadequate in some cases.

The purpose of this publication is to present a new method for the purification of histamine which is very suitable for application to large numbers of samples and which promises a high degree of selectivity. The essential features of this method are as follows. An aqueous, histamine-containing extract from tissue is extracted with *n*-butanol under conditions such that nearly all the histamine is removed from the aqueous phase in one extraction. The histamine is recovered from the butanol by means of a new cation exchange medium, cotton acid succinate. The histamine is eluted from the cotton acid succinate with a small volume of dilute hydrochloric acid and the eluate is neutralized with sodium hydroxide to give an isotonic solution suitable for bioassay.

*Effect of pH and Salt Concentration on Distribution of Histamine between Water and Butanol*—The first step in the development of this histamine purification procedure was to find a simple extraction procedure by which histamine could be separated from most of the other constituents of an aqueous extract of biological origin. We found that the distribution of histamine between water and butanol at a high pH could be shifted in favor of the butanol by the addition of a water-soluble salt to the system. Experiments were then set up to determine quantitatively the effect of both pH and salt concentration. Of several salts which were investigated, sodium sulfate was finally chosen because of its high water solubility, etc.

The data of Fig. 1 show the distribution of histamine between butanol and water (*a*) as related to sodium sulfate concentration in the aqueous phase at an optimum pH, and (*b*) as related to pH at an optimum or near

\* Preliminary report presented before the Federation of American Societies for Experimental Biology, Chicago, May 20, 1947.

optimum salt concentration These data show the per cent of histamine in the butanol phase at equilibrium when the water and butanol phases are at equal volumes The data are expressed in this manner rather than in terms of distribution coefficients in order to show more clearly the plateaus in the distribution curves The per cent of histamine which goes into the butanol phase changes very slightly with salt concentration above about 22 per cent or with pH above 12.5

In the routine procedure, described later, a mixture of sodium sulfate and trisodium phosphate is employed to yield an aqueous solution of about 22.5 per cent (weight to volume) sodium sulfate and about 3 per cent (0.17 M) trisodium phosphate, with a pH between 12.5 and 13 The salt mixture dissolves readily and produces optimum conditions for the extraction

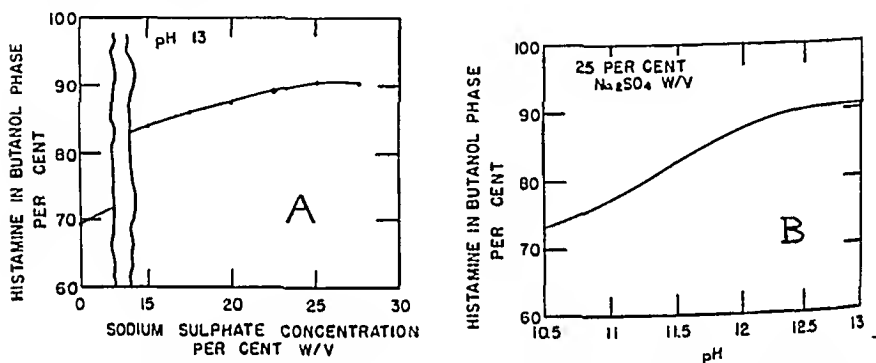


FIG. 1 The effect of salt concentration and pH on the distribution of histamine between water and butanol

The distribution of histamine between water and *n*-butanol, isobutanol, tertiary butanol, *n*-amyl alcohol, and isoamyl alcohol was determined at pH 13 and 25 per cent sodium sulfate concentration *n*-Butanol proved to be the alcohol of choice

*Recovery of Histamine from Butanol Solution by Means of Cotton Acid Succinate*—Following the development of conditions for the extraction of histamine from aqueous solution by means of butanol, a simple quantitative method of recovering histamine from the butanol was desired Such a procedure should yield, with a minimum of manipulations, an aqueous solution of physiological salt concentration which is free of organic solvents and in which the histamine is sufficiently concentrated for bioassay Perhaps the simplest procedure to satisfy these requirements would be the removal of histamine from the butanol by means of a cation exchange medium, or an adsorbent, from which the histamine could be eluted easily with a small volume of an aqueous solution The available cation exchange

media and histamine adsorbents were investigated. Several materials of both classes would remove histamine quantitatively from butanol, but the recovery of histamine was very unsatisfactory in all cases.

Finally, cotton acid succinate (hereinafter abbreviated as CS) was developed especially for this procedure. This material has the gross mechanical properties of cotton. It is produced by esterifying with succinic anhydride only a fraction of the free hydroxyls of cotton. For every hydroxyl group esterified, there is a free carboxyl which can function in cation exchange reactions. When a butanol solution of histamine is filtered through a small pad of CS, the histamine is quantitatively removed from the butanol. The CS can, without loss of the histamine, be washed with water until free of alcohol. The histamine can then be quantitatively

TABLE I  
*Recovery of Histamine from Water and Butanol Solutions by Means of Cotton Acid Succinate (CS)*

Solvent	Original histamine	
	In CS filtrate	In CS eluate
	per cent	per cent
Water*	0.6	102
	0.3	104
Butanol†	1.2	97
	1.4	97

\* 5 cc of aqueous histamine, 80  $\gamma$  per cc, 0.014 M  $\text{Na}_2\text{PO}_4$

† 5 cc of butanol, 67  $\gamma$  of histamine per cc

eluted from the CS with a small volume of dilute hydrochloric acid. The concentration of hydrochloric acid may be chosen so that neutralization with sodium hydroxide will yield an aqueous isotonic solution which may be assayed biologically for histamine.

The data of Table I illustrate the recovery of histamine from water or butanol solutions by means of CS. The solutions were filtered through 100 mg CS pads. The pads were washed well and then eluted with 1 cc of 0.4 N hydrochloric acid, followed by 1 cc of water. In these experiments, a chemical determination of histamine was employed. The data show that the recovery of histamine by cation exchange on CS is quantitative, within the accuracy of the histamine determination.

*Detailed Procedure for Purification of Histamine in Blood Plasma*—Thus far this procedure for the purification of histamine has been applied only to free histamine in animal blood plasmas. The details of the procedure are as follows:

1. In glass-stoppered test-tubes place 2 cc of plasma, dilute to 4.5 cc

with saline and add 1.2 gm of a salt mixture containing 6.25 gm of anhydrous sodium sulfate to 1 gm of trisodium phosphate monohydrate. Stopper the tubes and shake thoroughly.

2 To each test-tube add 5 cc of butanol, shake vigorously for a few seconds, let stand a few minutes and then shake briefly to break the protein gel. Shake vigorously in a mechanical shaker for  $\frac{1}{2}$  hour and then centrifuge at about 2000 R.P.M. for 15 to 20 minutes in a No. 1-SB International centrifuge with a No. 240 head.

3 Transfer 4.6 cc of butanol solution from each test-tube to a CS tube (Fig. 2, B, and Fig. 2, C) which contains 100 mg of CS packed tightly in

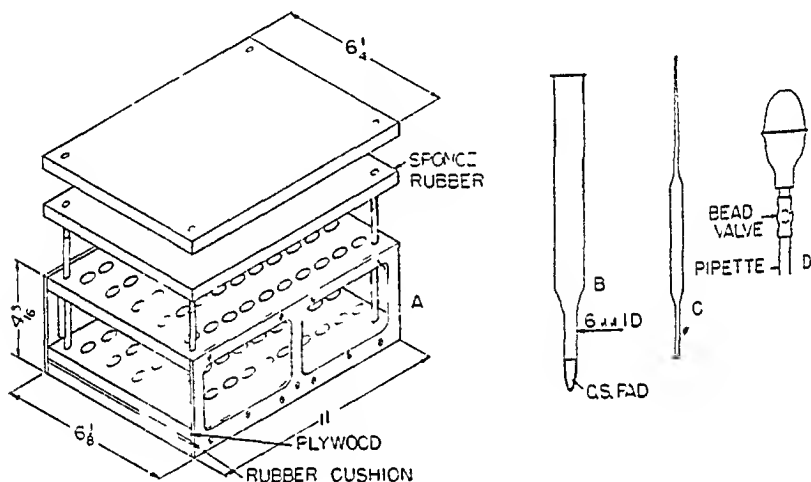


FIG. 2. A, special holder for shaking glass-stoppered test-tubes (the bottom, center, and top plates are made of  $\frac{1}{8}$  inch aluminum plate, the sides are of  $\frac{3}{16}$  inch aluminum plate), B, CS tube with 100 mg CS pad, C, glass weight which rests on CS pad to keep it firmly packed, D, pipetting bulb arrangement.

the 6 mm tube at the bottom. On account of the protein pad which is packed between the two liquid phases during centrifugation, the complete transfer of the butanol phase to the CS tube would be difficult, and 4.6 cc of butanol are nearly all that can be taken conveniently and consistently. The total volume of the butanol phase is 5.54 cc, therefore only 83 per cent of the histamine which goes into the butanol phase is transferred to the CS tube. The transfer of exactly 4.6 cc of butanol is most conveniently accomplished by means of a serological pipette (calibrated to the tip), to which is fitted a rubber bulb with a bead valve (Fig. 2, D). The bulb is attached to the pipette, the bead valve is opened and the bulb is exhausted. The bulb serves as a "vacuum reservoir" which will draw the liquid up into the pipette. The amount of liquid drawn up is easily regulated by the bead valve.

1 After the but mol has passed through the CS pad, wash the CS tube and pad with 3 cc of 95 per cent ethyl alcohol, followed by 3 cc of water. By means of a stirring rod, remove the last drop of water from the tip of the CS tube. Place a small test-tube or vial graduated to 2.5 cc under the tip of the CS tube and elute the histamine with 1 cc of 0.4 N hydrochloric acid, followed by 1 cc of water. Neutralize the eluate with 0.5 cc of 0.8 N sodium hydroxide and adjust the volume to 2.5 cc if necessary. This solution may be assayed biologically for histamine without further manipulation.

For the purification of large numbers of samples, the following time-saving devices may be employed.

In Step 1, a small metal cup may be used to measure the 1.2 gm aliquots of salt mixture. Slight deviations from the specified quantity of salt mixture are not of significant consequence.

For Step 2, a 5 cc Michlett automatic pipette was used in measuring out the butanol. A special rack was built (Fig. 2, A) in which 50 glass-stoppered test-tubes could be clamped securely during the mechanical shaking. This rack was clamped firmly in a precision equipoise heavy duty shaker, so that the tubes were lying lengthwise with the stroke of the shaker. The glass stoppered test-tubes were of the following specifications: Pyrex, 16 to 16.5 mm outer diameter, 126 mm over-all length, 104 mm length below the joint, standard taper 13/14 flat stoppers with the stopper head 18 mm in diameter. With tubes so designed, No. 356 metal cups with No. 355 three place trunnion carriers may be used in the centrifugation (Step 2). It has been found advisable to coat the test-tube stoppers with a thin film of vaseline before each experiment.

For Steps 3 and 4, 50 CS tubes have been used simultaneously. The CS tubes may be used almost indefinitely without changing the CS pads if suitable precautions are taken. The CS pad must not be treated with an excess of strong bases in aqueous solution, it should be washed thoroughly with water and then with 95 per cent alcohol immediately after each experiment.

With the equipment specified, one technician can purify the histamine from 50 plasma samples in 3 to 3½ hours.

Distribution studies in which histamine was determined chemically indicate that 93.3 per cent (average value) of the total histamine goes into the butanol phase in Step 2. Of this, 83 per cent is transferred to the CS pad in Step 3. With a 100 per cent recovery of histamine from the butanol by means of CS, the over-all yield of this procedure should be 77.4 per cent of the original total histamine. This figure can be increased to 95 per cent by the use of two butanol extractions instead of one (repeating Steps 2 and 3).

Table II shows the recovery, with one butanol extraction, of a known

quantity of pure histamine which had been added to rabbit, guinea pig, and dog blood plasmas. For these data the cat blood pressure assay for histamine was employed. This assay was performed on cats anesthetized with a phenopentobarbital mixture. Samples and histamine standards were administered via the femoral vein with the blood pressure recorded graphically from the carotid artery. Standardization was achieved by the injection of histamine in doses of 0.1 to 0.3  $\gamma$  until reproducible blood pressure drops were obtained. The fall in blood pressure produced by each sample was compared with histamine standard doses interspersed

TABLE II  
*Recovery of Histamine from Animal Blood Plasmas*

Plasma	Histamine added	Histamine recovered		Per cent recovery
		Calculated	Found	
	$\gamma$	$\gamma$	$\gamma$	
Rabbit	0		0.15	
	0		0.15	
	8	6.2	6.4	101
	8	6.2	6.4	101
	8	6.2	6.7	106
Guinea pig	0		0.32	
	0		0.25	
	8	6.2	6.4	98
	8	6.2	6.55	101
	8	6.2	6.70	103
Dog	0		0.0	
	0		0.0	
	8	6.2	6.4	103
	8	6.2	6.8	110
	8	6.2	6.65	107

throughout the experiment, and intermediate values were interpolated. The accuracy of this method averages within  $\pm 10$  per cent.

The data of Table II suggest that the above purification procedure is highly selective for histamine. There was no histamine activity extracted from the heparinized dog plasma "blanks." The small amount of histamine extracted from the rabbit plasma "blanks" represents only 5 to 8 per cent of the total histamine found in rabbit blood (2). On several occasions we have found, by means of the isolated guinea pig intestinal strip, that the histamine activity extracted from heparinized rabbit plasma could be nullified by small quantities of antihistamine drugs. The amount of histamine extracted from guinea pig plasma is approximately equal to the total histamine of guinea pig blood (2). Since in this case some hemolysis had occurred before the blood cells were separated from the

plasma, it might be expected that the plasma would contain nearly all of the blood histamine.

This procedure can be made even more selective for histamine by extraction of the plasma first with ether and then with butanol. The ether extract would contain no histamine and *should not be filtered through the CS pad*. Many drugs (amines), which might interfere with the histamine assay, are extracted from water by ether under these conditions and may thus be separated from histamine.

### *Materials and Methods*

The butanol, anhydrous  $\text{Na}_2\text{SO}_4$ , and  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$  were of reagent grade. The  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$  was obtained by drying reagent grade  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$  at  $100^\circ$  for 18 hours, then at  $300^\circ$  for 2 to 3 hours. The salts were finely powdered and mixed in a ratio of 6.25 gm of  $\text{Na}_2\text{SO}_4$  to 1 gm of  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ . In the study of histamine distribution between water and butanol as related to pH, phosphate buffers were used to obtain solutions of pH 10.5, 11, 11.5, and 12 (10). For pH 12.5 unbuffered solutions were made to 0.03 N with sodium hydroxide, for pH 13.0 1 N sodium hydroxide solutions were used.

Cotton acid succinate was prepared as follows. Fused sodium acetate (5 gm) and succinic anhydride (40 gm) were dissolved in 300 cc of glacial acetic acid. Cotton (10 gm) was immersed in this solution, a drying tube was attached to the flask and the temperature was maintained at  $100^\circ$  for 48 hours. The partially esterified cotton was filtered off, washed well with water, dilute hydrochloric acid, water, and finally with alcohol. The product was dried in a vacuum oven.

The chemical method used for histamine determination was a modification<sup>1</sup> of the method published by Baraud (11).

### SUMMARY

A histamine purification procedure has been given in detail. By this procedure free histamine may be purified for bioassay quickly and in good yield. The method is particularly designed for the purification of a large number of samples at one time.

The authors are grateful to Muriel Sproull for technical assistance and to William Campbell of the Engineering Department for the illustrations.

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# THE BIOLOGICAL SYNTHESIS OF RADIOACTIVE ADRENALIN FROM PHENYLALANINE

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(Received for publication, June 21, 1947)

Although there is indirect evidence that tyramine (1, 2), phenylethylamine, and phenylalanine (3) may be converted to adrenalin by surviving slices of adrenal medulla, the mechanism of these conversions is still obscure Vinet (4), while denying that tyramine is a biological precursor of adrenalin, was nevertheless able to demonstrate that the same tissue is able to convert  $\beta$ -(3, 4-dihydroxyphenyl)-ethylamine to adrenalin

Although there is no direct evidence that  $\beta$ -3, 4-dihydroxyphenylalanine is a normal metabolite in mammals, nevertheless there is a considerable body of evidence indicating that kidney (5), liver (6, 7), intestine (8), and pancreas (9) contain enzyme systems capable of converting this amino acid to its corresponding amine Medes (10) has reported the recovery of  $\beta$ -3, 4-dihydroxyphenylalanine from urine when large amounts of tyrosine were administered to an individual with tyrosinosis

In order to study the metabolic steps involved in the biological formation of adrenalin, radioactive DL-phenylalanine was synthesized and administered to rats with the hope that it would be possible to demonstrate its conversion to adrenalin in the intact animal

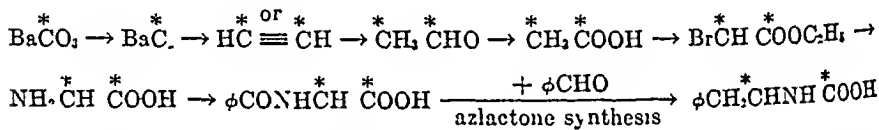
## EXPERIMENTAL

*Phenylalanine Labeled with  $H^3$* —2.0 gm of DL-phenylalanine were heated for 6 days at 50° with 8.5 gm of 84 per cent sulfuric acid made from  $SO_3$  and TOH For the separation of tritium-labeled phenylalanine the procedure of Moss and Schoenheimer (11) was employed 1.8 to 1.9 gm of recrystallized product were recovered

Analyses for tritium were carried out with a Geiger tube which could be evacuated and filled with the gas sample A standard mixture containing helium (60 mm), alcohol vapor (10 mm), and hydrogen (10 mm) was employed The instrument was calibrated with tritium prepared from standard solutions of TOH of known radioactivity For analysis of organic compounds, oxidation by heating with  $CuO$  was employed The evolved water was frozen and converted by means of metallic sodium at low temperature to hydrogen, which was then admitted into the Geiger tube The counts were calculated in terms of counts per minute per mole of

compound The error involved in the estimation is approximately 15 per cent

*Phenylalanine Labeled with C<sup>14</sup>*—For the synthesis of phenylalanine labeled with C<sup>14</sup> in the  $\alpha$ - or carboxyl carbons, doubly labeled glycine was prepared 10 gm of radioactive barium carbonate (containing a total radioactivity of  $1.2 \times 10^7$  counts per minute) were converted by way of barium carbide and acetylene into doubly labeled acetaldehyde (12) The acetaldehyde was oxidized by shaking with silver oxide to acetic acid, which was recovered by acidification and steam distillation The over-all yield of sodium acetate recovered by neutralization and evaporation was 40 per cent The sodium acetate was diluted with 3 times its weight of non-radioactive sodium acetate and the product converted to doubly labeled ethyl bromoacetate (12) By condensation with potassium phthalimide followed by subsequent hydrolysis, doubly labeled glycine was prepared (12) The glycine was converted to hippuric acid and condensed with benzaldehyde, yielding the azlactone (72 per cent yield) which was then converted with P and HI (13) to phenylalanine (69 per cent yield) The reactions involved are as follows



Radioactivity measurements were made with a thin mica window Geiger tube employing BaCO<sub>3</sub> plates Organic substances were oxidized by the method of Van Slyke and Folch (14), and the evolved CO<sub>2</sub> was trapped in saturated Ba(OH)<sub>2</sub> solution The washed, dried BaCO<sub>3</sub> was converted into a suitable plate, as described elsewhere (15), and measured The actual counts were corrected to the number of counts given by a plate of infinite thickness (16) and calculated as counts per mg of carbon per minute From this value the counts per mole of carbon per minute were calculated

*Animal Experiments with Tritium*—Tritiophenylalanine was fed as 1 per cent of the stock diet to a 150 gm rat for a period of 10 days The weight of phenylalanine consumed was 0.9 gm The animal was anesthetized with amytal and the adrenal glands were removed and promptly ground up with 2 N acetic acid containing SO<sub>2</sub> 50 mg of non-radioactive adrenalin were added as a carrier The suspension was heated for a few minutes at 90°, chilled, centrifuged, and the supernatant fluid and washes concentrated *in vacuo* under N<sub>2</sub> to dryness The temperature was maintained at 35° The residue was extracted several times with a few ml of ethanol, centrifuged, and the solution concentrated *in vacuo* under N<sub>2</sub> to dryness The

residue was dissolved in minimal dilute acetic acid containing  $\text{SO}_2$  and the solution made alkaline with  $\text{NH}_3$ . After chilling for several hours, crystalline adrenalin was centrifuged and recrystallized three times by solution in acetic acid followed by alkalinization with  $\text{NH}_3$ . 30 mg of colorless crystalline adrenalin were recovered, m p  $205\text{--}206^\circ$ , mixed m p  $205\text{--}205.5^\circ$  uncorrected  $[\alpha]_D = -51.0^\circ$  (1 per cent in 0.1 N HCl containing  $\text{SO}_2$ ).

*Experiments on Animals with  $\text{C}^{14}$ -Labeled Phenylalanine*—Three experiments were run with  $\text{C}^{14}$ -labeled phenylalanine. In the first experiment, three 100 mg portions of phenylalanine (total 300 mg) in dilute alkali (pH 8.5) were injected intraperitoneally at 2 hour intervals into a 250 gm rat. 6 hours after the first injection the animal was anesthetized with alyttil and the adrenals removed. 50 mg of non-radioactive adrenalin were added as a carrier and the isolation of adrenalin performed as described above. 30 to 35 mg of adrenalin were recovered after three recrystallizations, m p  $205.5\text{--}206^\circ$ , mixed m p  $205.5\text{--}206^\circ$  uncorrected  $[\alpha]_D = -50.1^\circ$ .

In the second experiment, a single injection of 100 mg of isotopic phenylalanine was given. The animal was anesthetized 24 hours later and adrenalin isolated in the usual manner. In this case 35 mg of adrenalin were used as a carrier and 20 to 25 mg recovered after three recrystallizations.

In the third experiment, two rats weighing 150 to 200 gm each were injected once daily for 3 days with 100 mg of radioactive phenylalanine. Each rat received, therefore, 300 mg of isotopic phenylalanine. On the 4th day the rats were anesthetized, the adrenals combined, and 50 mg of adrenalin added as the carrier. After three recrystallizations 30 to 35 mg of adrenalin were recovered. In this isolation, 10 to 20 mg of non-radioactive phenylalanine were dissolved in the acetic acid used for the third recrystallization of the adrenalin. The adrenalin was precipitated with  $\text{NH}_3$  and a fourth recrystallization performed in the presence of added non-radioactive phenylalanine, m p  $205.5^\circ$ , mixed m p  $205\text{--}206^\circ$  uncorrected  $[\alpha]_D = -50.8^\circ$ .

In order to be certain that the isolated adrenalin was not contaminated with traces of radioactive phenylalanine, a control experiment was performed. 10 mg of phenylalanine labeled with  $\text{C}^{14}$  were mixed with 50 mg of non-radioactive adrenalin. The mixture was dissolved in 2 N acetic acid and the adrenalin precipitated with excess ammonia. The adrenalin was recrystallized in the same way two more times. The product contained no demonstrable radioactivity.

### Results

In Table I are listed the results obtained with the animal to which phenylalanine labeled with tritium had been administered. The recovered

adrenalin was significantly radioactive, despite the large dilution resulting from the addition of 50 mg of carrier adrenalin to probably no more than 0.2 to 0.3 mg of endogenous adrenalin.

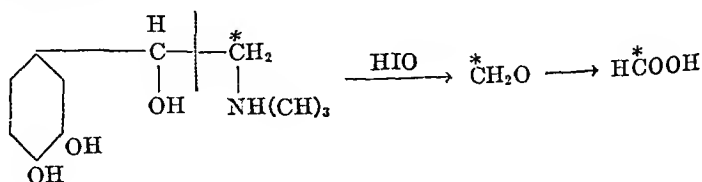
The adrenalin samples recovered after the administration of phenylalanine labeled with  $C^{14}$  were either very weakly radioactive or border line (Table I). As a further check on the radioactivity of these preparations, they were submitted to oxidation with a slight excess of periodic acid in 2.5 N  $H_2SO_4$ . In strong mineral acid, the formation of the usual oxidation products is minimal. After standing 4 hours, formaldehyde was steam-distilled and absorbed in cold alkaline hypiodite. After acidifica-

TABLE I  
Results of Administration of Tritium-Labeled and  $C^{14}$ -Labeled Phenylalanine\*

Tritium experiment	$H^3$ counts per mole per min
Administered tritio-phenylalanine	$7 \times 10^5$
Body water (blood)	$2 \times 10^5$
Recovered adrenalin	$1.2 \times 10^4$
$C^{14}$ experiments	$C^{14}$ counts per mole C per min
Administered phenylalanine ( $\alpha$ carbon)	$4.4 \times 10^5$
Recovered adrenalin, Experiment 1	$3.0 \times 10^3$
Formic acid C from adrenalin, Experiment 1	$3.7 \times 10^4$
Recovered adrenalin, Experiment 2	$4.0 \times 10^3$
Formic acid C from adrenalin, Experiment 2	$1.7 \times 10^4$
Recovered adrenalin, Experiment 3	$2.0 \times 10^3$
Formic acid C from adrenalin, Experiment 3	$1.3 \times 10^4$

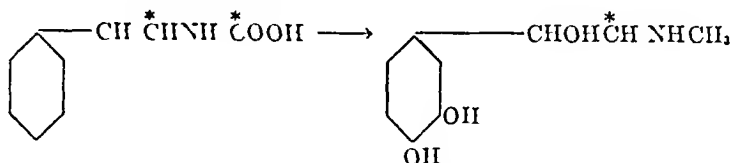
\* All counts are excess counts corrected for background

tion and removal of excess  $I_2$  with  $Na_2S_2O_3$ , the resulting formic acid was oxidized by boiling under a reflux with mercuric acetate in weak acetic acid. The evolved  $CO_2$  was trapped in barium hydroxide solution and tested for radioactivity. Essentially all of the radioactivity was found to be present in the formic acid carbon. No radioactivity could be detected in the remaining carbons.



## DISCUSSION

These results indicate that phenylalanine is a precursor of adrenalin. The location of the  $C^{14}$  in the isolated adrenalin demonstrates that, in addition to decarboxylation, the side chain remains attached to the benzene nucleus during the biologic synthesis of adrenalin.



These results suggest that the biosynthesis of adrenalin is not brought about by a condensation of benzaldehyde or *p*-hydroxybenzaldehyde with sarcosine, as suggested by Rosemund and Dornsaft (17).

The tritium content of the adrenalin recovered in the first experiment is higher than one would predict on the basis of dilution with carrier adrenalin and simultaneous loss of 4 out of the 5 tritium atoms of the benzene nucleus. These results suggest that either adrenalin turnover is rapid and the gland utilizes circulating phenylalanine or tyrosine efficiently, or that some tritium may have been present in the side chain and was partly retained during the conversion to adrenalin. The possibility exists that more than 1 tritium atom remains attached to the aromatic nucleus during this biosynthesis. The latter suggestion is rather surprising if one assumes that an orthoquinone is an intermediate in the formation of adrenalin from phenylalanine or tyrosine. However, Anchel and Schoenheimer (18) have reported a somewhat analogous situation in the case of deuteriocoprostanone labeled with deuterium on carbons located  $\alpha$  to the ketone group. When this material was administered to a man, coprosterol isolated from this individual contained appreciable amounts of deuterium.

From a quantitative point of view the results obtained in this series of experiments are not significant, since the radioactivity of the adrenalin was very low. The formic acid derived from the adrenalin samples was, however, considerably more radioactive. Qualitatively, it seems quite clear that the recovered adrenalin was radioactive and that the radioactivity is localized entirely in the terminal carbon of the side chain.

We wish to acknowledge our indebtedness to the David, Josephine, and Winfield Baird Foundation for financial aid and to express our thanks to Dr. Allen F. Reid for advice and help with the measurements of radioactivity. Our thanks are also due the American Cancer Society for financial aid.

## SUMMARY

DL-Phenylalanine labeled with  $C^{14}$  in the carboxyl or  $\alpha$ -carbons was synthesized from doubly labeled glycine. Upon administration to rats, this substance was converted to radioactive adrenalin which could be recovered by the addition of non-radioactive adrenalin as a carrier to extracts of the adrenals. The recovered adrenalin was radioactive and found to contain  $C^{14}$  localized in the terminal carbon of the side chain.

Tritium-labeled phenylalanine is similarly converted to adrenalin. The evidence suggests that the biological conversion not only involves decarboxylation of phenylalanine or one of its derivatives but that the resulting amino ethyl side chain remains attached to the benzene nucleus during the biological synthesis of adrenalin.

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# FRACTIONATION, ELECTROPHORESIS, AND CHEMICAL STUDIES OF PROTEINS IN SERA OF CONTROL AND INJURED DOGS\*

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(Received for publication, June 9, 1947)

The occurrence of unusual configurations in the electrophoretic patterns of plasma of experimental animals (1) and man (2) after injury and disease is of interest. In those conditions accompanied by trauma or destruction of tissue, the  $\alpha$ -globulin concentration increases. The present investigation is an attempt to determine the type of proteins responsible for the abnormal patterns seen in dogs after injury by vesicants, thermal injury, and turpentine.

Cohn and his associates (3) described methods for fractionating plasma proteins by using varying concentrations of ethanol at different hydrogen ion concentrations, low ionic strengths, and low temperatures. These procedures are valuable in concentrating and purifying plasma proteins. Advantage is taken of these new techniques to aid in the identification, purification, and chemical analysis of the proteins present in the sera of control and injured animals.

## Methods

Animals were injured with sulfur mustard, turpentine, and heat according to procedures previously described (1). The experimental dogs were anesthetized with sodium pentobarbital and exsanguinated from a cannula in the femoral artery. The blood was collected in centrifuge bottles and was allowed to stand at room temperature until the clot was well retracted. The serum was withdrawn immediately after centrifugation. An attempt was made to process the serum immediately but if this was impossible it was frozen and stored. Before the frozen serum was used, it was allowed to thaw slowly in a cold room at 2-5°.

A well insulated stainless steel tank containing 75 gallons of 30 per cent alcohol was used for maintaining low temperatures during the processing of sera and supernatants. The temperature of the alcohol-water mixture was usually maintained at -5° and the fluid was kept circulating by means of a large stirrer. Centrifugation was carried out between 0° and -10° in

\* The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and the University of Virginia. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

either a large capacity Servall angle centrifuge (type G/1), a high speed Servall angle centrifuge (type SS-1), or in a motor-driven Sharples clarifier centrifuge, in which all parts coming in contact with solutions were of stainless steel. The centrifuges were operated in constant temperature cabinets, and the temperature was registered on a Brown continuous recorder.

Fractionation of sera and supernatants was performed in stainless steel beakers. These containers were suspended in the bath by means of rigid supports and the solutions were stirred by variable speed motor-driven stainless steel stirrers. The volumes of sera were measured at room temperature and supernatants at  $-5^{\circ}$ . No correction was made for volume changes due to temperature. The ethanol concentrations of the solutions were adjusted by either 53.3 or 95 per cent ethanol. The ionic strengths of the solutions were adjusted by adding calculated amounts of sodium acetate-acetic acid buffer (3) or molar sodium chloride separately or dissolved in 53.3 per cent ethanol. Whenever the fraction was dissolved in water or saline for fractionation, the solvent was first brought close to the freezing point. Ethanol, ethanol-buffer, ethanol-NaCl, or salt mixtures were equilibrated to low temperatures in narrow necked graduated cylinders immersed in the bath and were added to the serum or the supernatant at the rate of 20 to 30 ml per minute through a siphon by means of a constant air pressure.

The pH of the solutions was usually adjusted with an acetate buffer of pH 4.0 and an ionic strength of 0.4. The successive steps in most fractionation procedures require increased hydrogen ion concentrations. To determine the amounts of buffer to be added, a few ml were titrated with an acetate buffer of pH 4.0 with an ionic strength of 0.016. Molar sodium bicarbonate was used whenever the pH was to be increased.

Immediately after the precipitation of a fraction, an aliquot was dissolved in a barbiturate-NaOH buffer (pH 8.6, ionic strength 0.1) and was dialyzed against 2 liters of this same buffer for 3 days at  $2-5^{\circ}$ . Electrophoresis was carried out in the Tiselius apparatus according to Longworth's modification of the schlieren method (4). A long single section cell of 11 ml capacity was used. Electrophoresis was usually allowed to continue for 7200 seconds at  $2^{\circ}$  with a potential gradient of 5 to 8 volts per cm. The mobility calculations were based on the distance from the salt effect peak ( $\delta$ ) to the peaks of the respective components. The descending pattern was used for computing mobility, but in some instances it was necessary to use the ascending pattern. Measurements made from the initial boundaries could not be used because of the large variations in values.

Protein fractions, dried to constant weight over  $P_2O_5$  *in vacuo*, were



extracted by two different procedures for the analysis of total lipid C (5), total and free cholesterol (6), and phospholipide (7). Procedure I involved two extractions with an absolute ethanol-ethyl ether mixture (1:1) under reflux. Because of poor yields in fractions having low lipid contents, it was suspected that the boiling temperature (about 40°) of the extraction mixture was too low. Satisfactory extraction of total lipid C, total cholesterol, and phospholipide was obtained by first extracting the dried protein with absolute ethanol for 12 hours under a reflux, which was followed by a second extraction with an absolute ethanol-ethyl ether mixture (1:1) (Procedure II). Further investigation indicated that the free cholesterol values were too low by this latter method. The procedures recommended for the determination of free and total cholesterol in plasma by Sperry (6) were adapted to the wet fractions (Procedure III). The data are expressed in terms of dry weight.

Cholesterol was converted to cholesterol carbon with the factor 0.839. The fatty acid bound to cholesterol was estimated by multiplying the ester value by 0.67 and its carbon content was obtained by multiplying by 0.759. Phospholipide phosphorus was converted to phospholipide by using the factor 23.7 and the carbon was obtained by multiplying by 0.648.

Hexosamine was determined in a number of fractions by adapting the method of Palmer, Smyth, and Meyer (8) to the Evelyn colorimeter. Choline was determined on the alcohol-ether extract by Engel's (9) method. Total nitrogen was analyzed by the micro-Kjeldahl method.

### Results

During the course of these investigations, a large number of mobility data were collected for dog sera. The mean values and the standard errors for each protein component are shown in Table I and serve as the guide for identifying the various proteins in the isolated fractions.

Four major fractions were obtained from the serum of normal dogs by fractionation according to Method 6 described in detail by Cohn and associates (3). While studying the serum of injured dogs it was found that two new fractions designated as Fractions IV and IV-4f were present after the subcutaneous injection of turpentine or the application of bis( $\beta$ -chloroethyl) sulfide to the skin. All sera were consequently fractionated according to the outline shown in Diagram 1. Electrophoretic patterns for five of the six fractions are shown in Figs. 1 and 2, solutions of Fractions IV-4 were usually too turbid for analysis.

The data for the percentage distribution of proteins in whole serum or plasma which were fractionated are presented in Table II. An increase in the percentage distribution of the  $\alpha$ -globulins and a decrease in the albumin were constantly observed in the sera of these injured dogs. Elec-

trophoretic analyses of dog sera were far from satisfactory, since many of the patterns were not clearly defined. At times it was necessary to consider a group of proteins as a single entity.

Each of the main fractions was fractionated and the methods used (Diagrams 1 to 6) and the percentage distribution of proteins (Tables I to VII) are detailed.

Fraction II+III was fractionated according to the procedure described by Cohn and associates (10) in their Method 9 and is outlined in Diagram 2.

The two fractions, Nos II+III-W and III-0, were first precipitated and each was subjected to further fractionation. Fraction II + III-W yielded Fractions III, III-3, II-3, and II-1,2. Further fractionation of Fraction III yielded Fractions III-2 and III-1. Thus Fraction II + III-W

TABLE I  
*Mobilities of Components in Whole Serum\**

Albumin	Globulins				
	$\alpha_1$	$\alpha_2$	$\beta_1$	$\beta_2$	$\gamma$
Descending boundaries (25 dogs)					
$7.2 \pm 0.04^\dagger$	$5.6 \pm 0.08$	$4.6 \pm 0.08$	$3.3 \pm 0.08$	$2.8 \pm 0.08$	$1.8 \pm 0.03$
Ascending boundaries (8 dogs)					
$7.8 \pm 0.10$	$5.8 \pm 0.14$	$5.0 \pm 0.12$	$3.8 \pm 0.09$	$3.1 \pm 0.12$	$2.0 \pm 0.03$

\* In barbital buffer at pH 8.6, ionic strength 0.1, and temperature 2°, expressed as  $\text{cm}^2 \text{ volt}^{-1} \text{ sec}^{-1} \times 10^{-5}$ .

† Standard error

yielded five subfractions. Procedures developed in this laboratory for fractionating Fraction III-0 into two main subfractions (Nos III-0A and III-0B) are outlined in Diagram 3. The yield of Fraction III-0A is consistently increased in injured animals.

Fraction II + III and its subfractions contained all of the  $\gamma$ - and most of the  $\beta$ -globulins (Table III). The small amounts of  $\alpha$ -globulin and albumin present may be due in part to occlusion of the supernatant. The effect of injury on the percentage distribution of proteins in the main fraction is difficult to assess because of the large variation in values for the control and experimental animals.

Fraction II-3 is made up almost entirely of  $\beta$ - and  $\gamma$ -globulins. The percentage distribution of  $\beta$ -globulins increases in this fraction obtained from dogs injured by turpentine. Fraction II-1,2 is an electrophoretically pure  $\gamma$ -globulin in control serum, a small amount of  $\beta$ -globulin is present

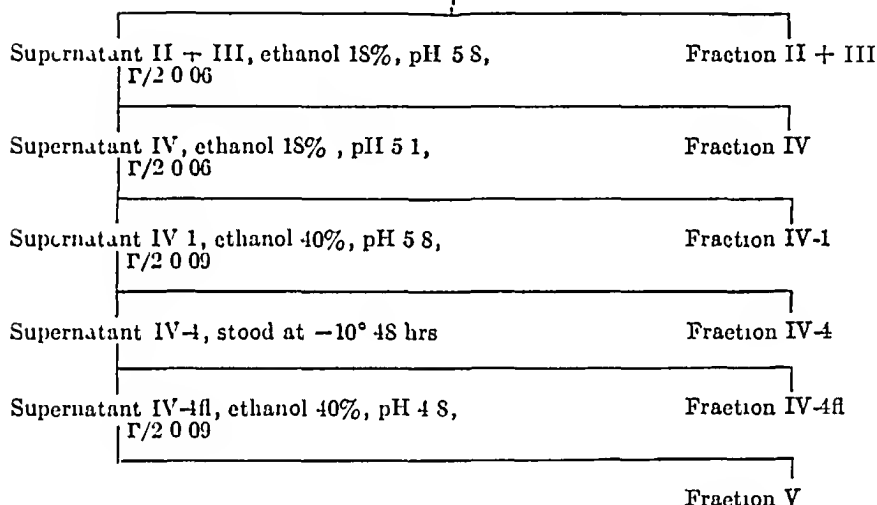
in the fraction of the turpentine-injured dog. A comparison of the mobilities and of the conditions for precipitation of the  $\gamma$ -globulins of Fractions II-3 and II-1,2 indicates that these are probably two distinct proteins in

DIAGRAM 1

*Major Fractionation of Serum*

Serum

Ethanol 25%,  
pH 6.8,  
 $\Gamma/2.008$



(1) The serum is brought to a final ethanol concentration of 25 per cent by adding 53.3 per cent ethanol. (2) Fraction IV, when present, is precipitated from Supernatant II + III by first lowering the ethanol concentration to 18 per cent with water and then adjusting the pH to 5.8 with acetate buffer and allowing to settle for 8 hours. (3) Adjustment of the supernatant to pH 5.1 yields Fraction IV-1 after standing at  $-5^{\circ}$  for 12 hours. (4) Fraction IV-4fl is precipitated from Supernatant IV-4 after standing in a tall cylinder in the bath at  $-8^{\circ}$  to  $-10^{\circ}$  for 48 hours. The flocculent precipitate is collected after siphoning off the supernatant and is characterized by a low density. In order to obtain a well packed precipitate, it is necessary to centrifuge this material for an hour at 12,000 R.P.M. in a high speed angle centrifuge at  $-10^{\circ}$ . (5) Fraction IV-4 yields solutions too turbid for electrophoretic analysis.

dog serum. Fraction III-1 is composed of  $\beta$ - and  $\gamma$ -globulins. Fraction III-2 consistently yields solutions too turbid for electrophoretic analysis, but the few available data indicate that it is composed chiefly of  $\alpha$ - and  $\beta$ -globulins.

Fraction III-0 is difficult to analyze owing to the turbidity of the solu-

tion From the available data, it may be said that  $\alpha$ - and  $\beta$ -globulins are the chief components of this fraction

Fraction IV is obtained in small amounts from the sera of most of the injured dogs (Table VII) It is composed chiefly of  $\alpha$ - and  $\beta$ -globulins

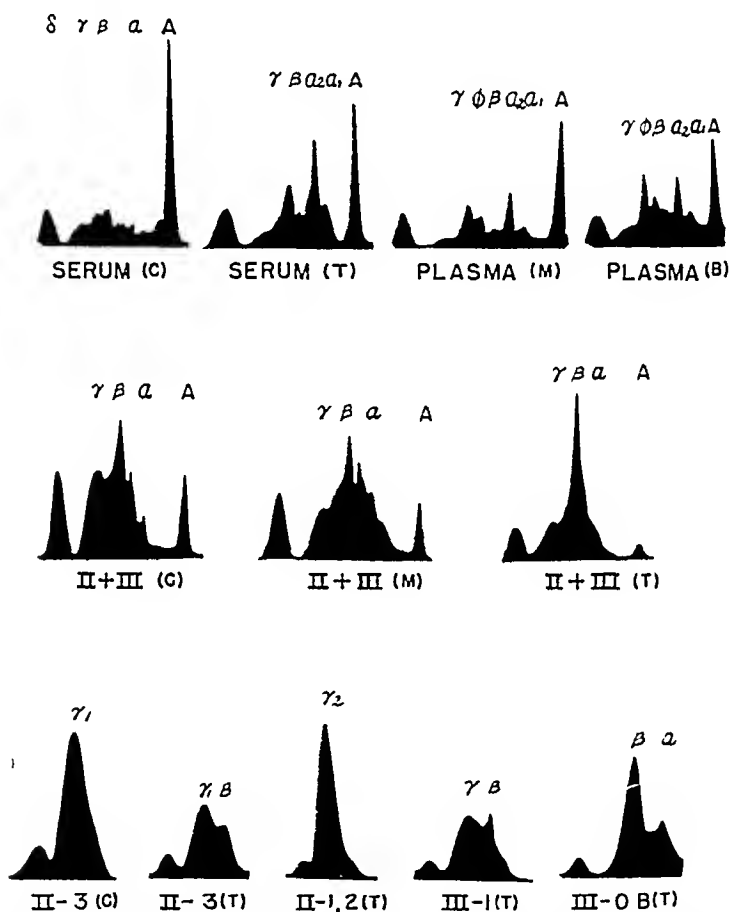


FIG 1 Ascending electrophoretic patterns of dog serum, plasma, and fractions in barbiturate buffer, pH 8.6,  $\Gamma/2$  0.1 at 2° C, control, T, turpentine, M, mustard, B, burned

(Table IV) Insufficient amounts are available for subfractionation and it was combined with Fraction IV-1 for fractionation

Fraction IV-1 is composed chiefly of  $\alpha$ - and  $\beta$ -globulins (Table IV), the percentage distribution does not appear to be affected by injury This fraction contains a new component having a mobility between 6.0 and 6.5  $\times 10^{-5}$  This protein is classified as  $\alpha_0$  and has a mobility between that of

albumin and  $\alpha_1$ -globulin. Two subfractions, Fractions IV-1A and IV-1B (Diagram 1), are composed chiefly of  $\alpha$ -globulins. The fast component  $\alpha_2$  does not appear in the subfractions.

Fraction IV-1 yields solutions which are usually too turbid for electrophoretic analysis, but its subfractions are satisfactory for study (Diagram 5, Table V). Thus far, Fraction IV-1B has been separated only from the sera of three dogs subjected to turpentine injections. Fraction IV-4 of two burned dogs was subfractionated without obtaining Fraction IV-4B. Insufficient material was available after mustard treatment to determine the presence of this fraction.

TABLE II  
*Electrophoretic Analyses of Dog Sera and Plasma Used for Fractionation*

No of samples	Treatment*	Per cent distribution				
		Albumin	$\alpha$	$\beta$	$\gamma$	$\phi$
3	N	46	20	26	8	
4	T	31	42	24	3	
2	B	31	30	24	7	(16)†
1	M	38	27	16	4	15

\* N, control, T represents Runs 1, 7, and 35, 0.5 ml of turpentine injected subcutaneously into six different sites on the back, the dogs sacrificed 3 days later, B, dogs clipped under deep anesthesia, dipped into hot water at 75° for 6 seconds, and sacrificed 4 and 7 days later, M, cutaneous application of 40 mg per kilo of bis-( $\beta$ -chloroethyl) sulfide, dogs sacrificed 4 days later (Run 70).

† Value for one dog.

Excellent electrophoretic patterns obtained for Fraction IV-4B in two dogs injured with turpentine showed different configurations (Fig. 2). One appeared to be composed of a fairly homogeneous protein with a mobility of  $5.7 \times 10^{-5}$ . The predominant proteins of the other showed two distinct peaks having mobilities of 5.4 and  $5.0 \times 10^{-5}$  and could be classified as  $\alpha$ -globulins.

Fraction IV-4C is composed principally of  $\alpha$ -globulins and is the largest subfraction of Fraction IV-4. It is obtained in increased amounts from the sera of the injured dogs. Fractions IV-4D and IV-4E contain most of the albumin present in the main fraction. An appreciable amount of  $\alpha$ -globulin is present in Fraction IV-4E, obtained from the serum of injured animals.

Fraction IV-4f can be separated from the serum of dogs treated with mustard and turpentine. It was not recovered from the sera of the control or burned animals. The mobility of the main component is about  $5.0 \times 10^{-5}$  and it is therefore classified as an  $\alpha_2$ -globulin.

# DIAGRAM 2

## Subfractionation of Fraction II + III

Fraction II + III dissolved in 7.5 times its weight of H<sub>2</sub>O

Ethanol 20 per cent,  
pH 7.2

Supernatant II + III-W, ethanol 25%,  
pH 5.7 (Ca 1c)

Fraction II + III-W

Fraction III-0

The aqueous solution of Fraction II + III is brought to 20 per cent ethanol by adding 53.3 per cent ethanol and to pH 7.2 by adding a small amount of NaHPO<sub>4</sub> to precipitate Fraction II + III-W. Fraction III-0 is precipitated after adjusting Supernatant II + III-W to pH 5.7 with calcium acetate buffer (pH 4.0) and the ethanol concentration to 25 per cent with 95 per cent ethanol. The precipitate settled for 48 hours at -5°. After separating Fraction III-0 in the angle centrifuge, the supernatant is practically protein-free. If the Sharples centrifuge is used for this separation, a turbid supernatant is obtained which contains appreciable amounts of protein. This residual protein could best be precipitated by adjusting the ethanol to 40 per cent and centrifuging off in the angle centrifuge. Whenever this is done, the precipitate is pooled with Fraction III-0. Fraction II + III-W yields a clear solution in the barbiturate buffer, while Fraction III-0 is usually too turbid for electrophoretic analysis.

Fraction II + III-W dissolved in 11 times its weight of H<sub>2</sub>O

Ethanol 6.9%,  
pH 5.4, I/2 0.005

Supernatant III, ethanol 17%, pH 5.2, I/2 0.015

Fraction III

Supernatant III-3, ethanol 17%, pH 5.2, I/2 0.05

Fraction III-3

Supernatant II-3, ethanol 22%, pH 7.5, I/2 0.05

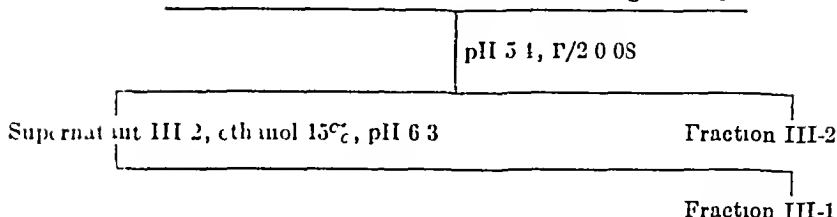
Fraction II-3

Fraction II 1,2

Fraction II + III-W, dissolved in water, is adjusted to pH 5.4 and ionic strength of 0.005 with acetate buffer and to 6.9 per cent ethanol with an equal volume of 13.8 per cent ethanol. The precipitate (III) is removed by centrifugation at -2°. Fraction III-3 is obtained by adjusting the pH of Supernatant III to 5.2 with acetate buffer, the ionic strength at 0.015 with NaCl, and the ethanol to 17 per cent with 53.3 per cent ethanol. Fraction II-3 is precipitated by increasing the ionic strength of Supernatant II-3 from 0.015 to 0.05 with NaCl. Fraction II-1,2 is removed by centrifugation after the supernatant is adjusted to pH 7.5 with NaHCO<sub>3</sub>, and the ethanol concentration increased to 22 per cent by addition of 95 per cent ethanol. Fraction III, which is turbid in aqueous solution, is further fractionated.

DIAGRAM 2—*Concluded*

Fraction III dissolved in 11.5 times its weight of H<sub>2</sub>O

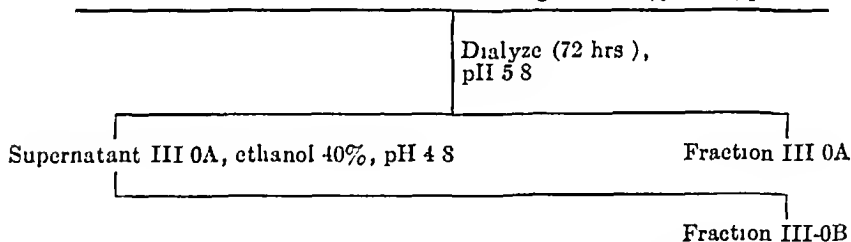


An aqueous solution of Fraction III is adjusted to pH 5.4 and an ionic strength of 0.08 with acetate buffer. This mixture is stirred for several hours and left for 8 hours at 0° and Fraction III-2 is centrifuged off. Fraction III-1 is precipitated and removed by centrifuging after the supernatant is adjusted to pH 6.3 with NaHCO<sub>3</sub> and to 15 per cent ethanol by adding an equal volume of 30 per cent ethanol. Solutions of Fraction III-2 are always too turbid for electrophoresis, solutions of Fraction III-1 are usually clear.

## DIAGRAM 3

*Subfractionation of Fraction III-0*

Fraction III-0 dissolved in 10 times its weight of 0.85% NaCl, pH 7.0



Precipitate III-0 is stirred for several hours with physiological saline, adjusted to pH 7.0 with NaHCO<sub>3</sub> at 0° until dissolved. This solution is dialyzed in a cellophane tubing for 72 hours at 5° against running tap water. The dialyzed solution is adjusted to pH 5.6 to 5.8 with pH 4.0 acetic acid acetate buffer and allowed to remain at 0° for about 12 hours. Fraction III-0A is removed by centrifuging at 0°. To Supernatant III-0A, 53.3 per cent ethanol which contains acetate buffer is added to give a final pH of 4.8 and an ethanol concentration of 40 per cent, Fraction III-0B is precipitated and centrifuged at -5° immediately.

Fraction V, which is chiefly albumin, contains appreciable amounts of  $\alpha$ -globulin in the controls and larger amounts in injured animals. Procedures described by Cohn (3) (Diagram 6) for the preparation of pure albumin are not successful in separating the  $\alpha$ -globulins from albumin.

The yields for various fractions are shown in Table VII. The increase in the  $\alpha$ -globulin-rich fractions (Nos. IV-4 and IV-4f) and the decrease in the albumin-rich fraction (No. V) parallel the changes noted in the electro-

phoretic patterns of the sera of injured animals After the subfractionation of Fraction II + III from serum of turpentine-injured dogs, there was a decrease in the yields for  $\gamma$ -globulin (Fractions II-3 and II-1,2) and a striking increase in the  $\alpha$ - and  $\beta$ -globulins (Fraction III-0)

TABLE III  
*Electrophoretic Analyses of Fraction II + III and Subfractions*

No of samples	Treatment*	Fraction No	Per cent distribution			
			Albumin	$\alpha$	$\beta$	$\gamma$
2	N	II + III	13	18	48	21
3	T	"	8	15	60	17
2	B	"	12	19	54	15
1	M	"	8	32	50	10
1	M	II-3		2	17	81
2	T	"		2	38	60
1	N	II-1, 2				100
1	T	"			8	92
1	N	III-1		10	44	46
1	T	"			57	43
1	"	III-0	8	53	27	12

\* See Table II

TABLE IV  
*Electrophoretic Analyses of Fraction IV, and Fraction IV-1, and Subfractions*

No of samples	Treatment*	Fraction No	Per cent distribution				
			Albumin	$\alpha_2$	$\alpha$	$\beta$	$\gamma$
2	T	IV	18		50	32	2
2	B	"	20		50	30	
1	M	"	13		64	21	
2	N	IV-1	24	19	41	16	
1	B	"	17		71	12	
2	M	"	16	24	45	15	
1	N	IV-1A	8		64	28	
1	T	"	7		77	16	
2	"	IV-1B	6		68	26	
1	"	IV-1C	60		40		

\* See Table II

The data for lipid and nitrogen analyses for two control and two turpentine-injected dogs are shown in Table VIII The results for Dogs 7 and 8 are limited, owing to the small amount of serum The lipides of these sera were extracted according to Procedure I The sera for Runs 34 and

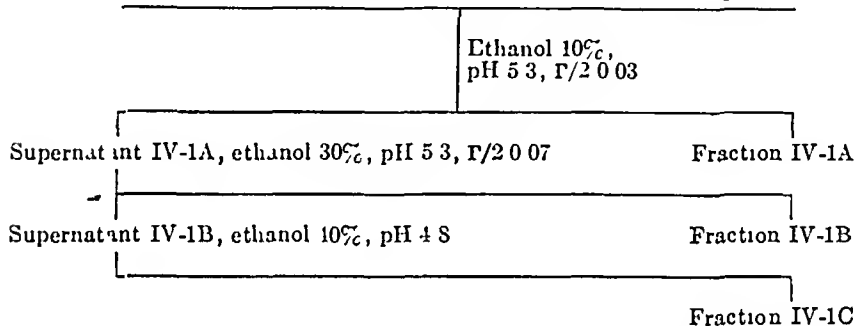


35 represent pooled samples from control and injured dogs, obtained through the courtesy of the Medical Division, Edgewood Arsenal. These animals were not in good shape at the time of the experiment and the sera were usually turbid on arrival in this laboratory. The results for these last two runs may not be typical for healthy control or turpentine-injected dogs. Lipide extractions for Runs 34 and 35 were performed according to Procedures II and III.

#### DIAGRAM 4

##### *Subfractionation of Fraction IV-1*

Fraction IV-1 dissolved in 6 times its weight of H<sub>2</sub>O at pH 7.0



Fraction IV-1 is dissolved in water, and brought to pH 7.0 with NaHCO<sub>3</sub> with stirring at 0°. The ionic strength is raised to 0.03 with NaCl, pH lowered to 5.3 with acetate buffer, and ethanol adjusted to 10 per cent with 53.3 per cent ethanol. The resulting precipitate (Fraction IV-1A) is separated by centrifuging. The ionic strength and ethanol concentration of Supernatant IV-1A are changed by the same procedures outlined above. Fraction IV-1B precipitates immediately and is removed by centrifuging. By lowering the pH of Supernatant IV-1B to 4.8 with acetate buffer and raising the ethanol concentration to 40 per cent with 95 per cent ethanol, Fraction IV-1C is removed by centrifuging. (In Run 7, Fractions IV and IV-1 were combined and fractionated as above.)

*Fractions II + III*—The increase in the total lipide C on the whole sera of turpentine-injected dogs appears to be due chiefly to changes in Fraction II + III. Subfractionation of Fraction II + III in sera of injured animals yields increased amounts of Fraction III-0 which contain larger concentrations of lipide C than was found in the controls. The lipide C concentrations of the  $\gamma$ -globulin-rich Fractions II-1,2, II-3, and III-1 are not changed appreciably after injury.

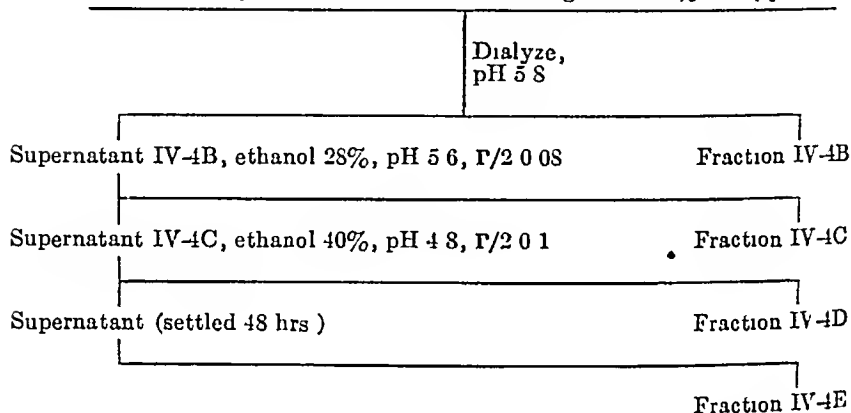
*Fraction IV-1*—The lipide concentrations of Fractions IV-1A and IV-1B are not appreciably affected by injury. Although the distribution of the  $\alpha$ - and  $\beta$ -globulins is about the same in these two fractions, the lipide concentrations are different. The lipide concentration of Fraction IV-1C is greater in the serum from the controls than from injured animals.

*Fraction IV-4*—Fraction IV-4A, which was insoluble and was obtained during the first subfractionation procedures (Runs 7 and 8), contained 5.1 and 6.6 per cent total lipide C in sera from control and turpentine-treated dogs, respectively

# DIAGRAM 5

## Subfractionation of Fraction IV-4

Fraction IV-4, dissolved in 12 times its weight of 0.85% NaCl, pH 7.0



Fraction IV-4 is dissolved by stirring for several hours in saline brought to pH 7.0 with NaHCO<sub>3</sub> at 0°. In a few experiments an insoluble gelatinous precipitate is formed on standing at 0° and is called Fraction IV-4A. The solution of Fraction IV-4 is dialyzed against running tap water maintained at 5° for 72 hours and is then adjusted to pH 5.8 with acetate buffer. Fraction IV-4 obtained from the serum of severely injured animals immediately yielded a flocculent precipitate which packed well on centrifugation. This precipitate designated as Fraction IV-4B is not found in normal dog serum. To the supernatant of Fraction IV-4B, sufficient NaCl is added to give an ionic strength of 0.08, the ethanol concentration is brought to 28 per cent and pH 5.6 with 53.3 per cent ethanol containing the acetate buffer. This mixture is stirred for several hours at -5° and kept at that temperature overnight. Fraction IV-4C is removed by centrifuging. The ionic strength of the supernatant of Fraction IV-4C is raised to 0.1 with NaCl, the pH lowered to 4.8 with acetate buffer, and the ethanol concentration increased to 40 per cent with 95 per cent ethanol. A precipitate (Fraction IV-4D) forms immediately on mixing and is centrifuged down at once. If the supernatant of Fraction IV-4D is allowed to stand at -5° for 48 hours, a precipitate (Fraction IV-4E) settles out from the sera of severely injured dogs.

Data for two samples of Fraction IV-4B, found only in the serum of turpentine-injected dogs, are presented. The fraction obtained in Run 7 is characterized by a low nitrogen content (3.6 per cent) and a very high total lipide C concentration (51.0 per cent). This lipide C could be almost completely accounted for as phospholipide and cholesterol. The fraction

TABLE V  
Electrophoretic Analyses of Subfractions of Fraction IV-4

No of samples	Treatment*	Fraction No	Per cent distribution		
			Albumin	$\alpha$	$\beta$
2	T	IV-4B	5	91	4
1	N	IV-4C	14	76	10
2	T	"	15	79	6
1	M	"	21	68	11
2	T	IV-4D	51	48	1
2	B	"	33	57	10
1	N	IV-4E	87	13	
1	T	"	17	53	
1	B	"	55	45	
2	T	IV-4FL	22	76	2

\* See Table II

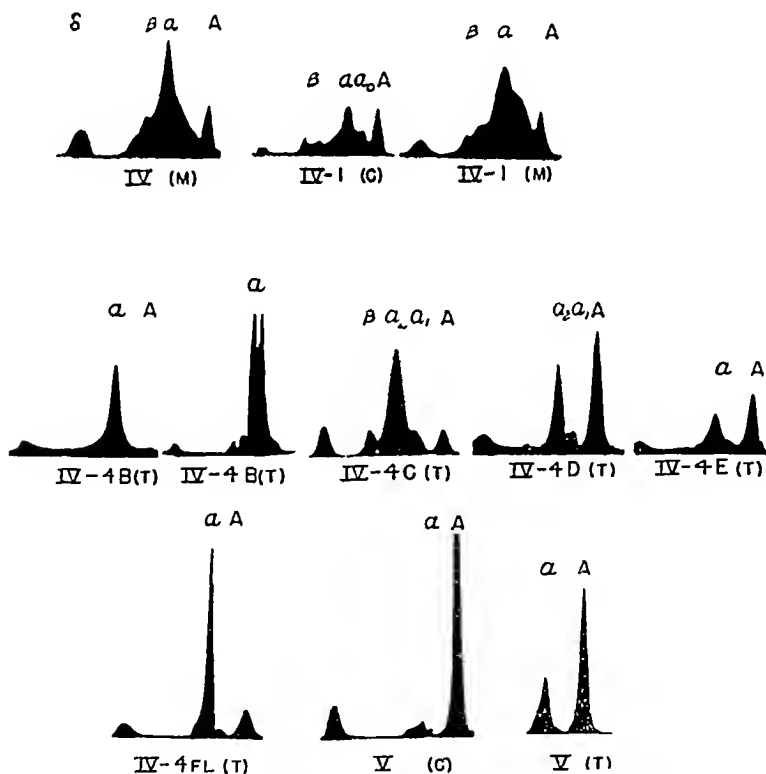


FIG 2 Ascending electrophoretic patterns of fractions of dog sera in barbiturate buffer, pH 8.6,  $\Gamma/2.0$  l at  $2^{\circ}$  C, control, T, turpentine, M, mustard, B, burned

of Run 35 has a total lipide C concentration of 34.4 per cent and a nitrogen concentration of 7.2 per cent. Only 63 per cent of this lipide C could be accounted for and most of this is due to phospholipide. The effect of

TABLE VI  
*Electrophoretic Analyses of Fraction V and Its Subfraction*

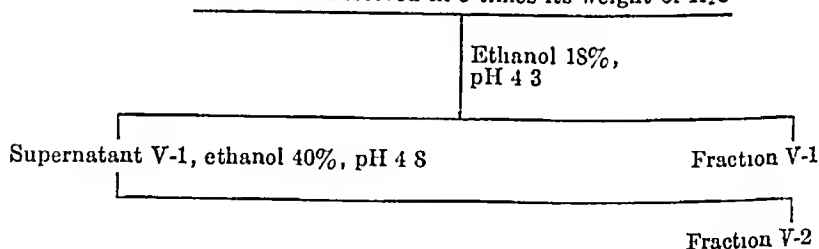
No. of samples	Treatment*	Fraction No.	Per cent distribution		
			Albumin	$\alpha$	$\beta$
2	N	V	82	16	2
1	T	"	64	36	
1	B	"	65	35	
1	M	"	78	28	
1	N	V-1	75	24	1
1	T	"	65	35	
1	N	V-2	88	12	
1	T	"	78	22	

\* See Table II.

DIAGRAM 6

*Subfractionation of Fraction V*

Fraction V dissolved in 6 times its weight of H<sub>2</sub>O



The procedures for purification of the albumin are similar to those described by Cohn *et al.* (3). Fraction V is dissolved in water at 0°, the pH adjusted to 4.3 with acetic acid, and the ethanol concentration raised to 18 per cent. Fraction V-1 is precipitated at once. Supernatant V-1 is adjusted to pH 4.8 and an ethanol concentration of 40 per cent and Fraction V-2 is obtained. Fraction V-1 usually yields a turbid solution while Fraction V-2 usually yields a clear solution.

injury on the lipide constituents of Fraction IV-4C is not striking or consistent in the two experiments.

*Fraction IV-4C*, which is the principal fraction of Fraction IV-4, is comparatively rich in lipide. Injury does not cause consistent changes in the lipide concentration or distribution.

*Fraction IV-4f*—The results of the lipide analyses of two different frac-

tions obtained in Runs 7 and 35 are given. The lipid C concentrations are 2.1 and 11.6 per cent respectively. Practically all of this lipid is accounted for as phospholipid.

*Fraction V*—The lipid C concentration of this albumin-rich fraction is diminished after injury. Further subfractionation yields fractions which are cholesterol-free and low in phospholipid content.

The results in Table VIII show consistent changes for a number of fractions in the two groups. The  $\gamma$ -globulin-rich Fractions II-1,2 and II-3 are decreased in amount but the low lipid concentrations are not changed after injury. Fraction III-0, composed chiefly of  $\alpha$ - and  $\beta$ -globulins, shows absolute increases in lipids due to increased yields and concentra-

TABLE VII

*Yields of Major Fractions and Subfractions of Dog Sera Expressed in Dry Weights per 1000 ml of Whole Serum*

Fraction No	Control Run 34	Turpentine Run 35	Turpentine, Run 7
	gm	gm	gm
II + III	26.0	28.0	25.0
IV	None	None	1.8
IV-1	4.4	5.0	1.3
IV-1	6.8	14.0	13.0
IV-4B	None	3.0	3.0
V	21.0	16.0	12.0
II-3	6.2	0.7	
II-1, 2	2.3	0.8	
III-2	1.4	3.4	
III-1	4.1	2.7	
III-0	9.0	15.0	

tions in the sera of turpentine-injected dogs. The two samples of Fraction IV-4B, found only in the sera of turpentine-injected dogs, have the highest concentration of lipid constituents recorded in these experiments. The absolute amounts of lipids of the  $\alpha$ -globulin-rich Fraction IV-4C are increased in the injured animals, chiefly because of the greater yields. The lipid concentrations and content of Fraction V are decreased in the sera of injured dogs.

The inability to account for all of the lipid C in most of the fractions as cholesterol, cholesterol fatty acids, and phospholipid is probably due to the presence of undetermined fatty acids or neutral fat.

*Sulfur Mustard Injury*—The changes in the lipid components for three main fractions of the serum of a dog treated with bis( $\beta$ -chloroethyl) sulfide are shown in Table IX. Sulfur mustard was applied (40 mg per kilo) under sodium pentobarbital anesthesia to the shaved back and the animal

was sacrificed 4 days later. The total lipide C and total cholesterol concentrations were increased in Fractions II + III and IV-4 and de-

TABLE VIII

*Nitrogen and Lipide Analyses of Protein Fractions in Control and Turpentine Injected Dogs*

Fraction No	Nitro- gen	Total lipide C	Total choles- terol	Free cholesterol	Phospholipide	C of lipide constituents accounted for*	Nitro- gen	Total lipide C	Total choles- terol	Free cholesterol	Phospholipide	C of lipide constituents accounted for*
Control Run 8							Turpentine Run 7					
	gm per cent	gm per cent	gm per cent	per cent	gm per cent	per cent	gm per cent	gm per cent	gm per cent	per cent	gm per cent	per cent
II + III	14.3	2.4	0.45		2.0		13.1	6.6	2.01		3.3	
II-3							15.0	1.2	0.14		1.4	
II-1,2							14.1	0.5	0.00		0.8	
III-1							14.0	1.6	0.50		0.8	
III-0							12.6	12.8	3.90		9.2	
IV-1A		12.4	4.76		10.4		12.2	12.1	3.36		8.9	
IV-1B	12.5	7.9	1.98		4.7		12.2	5.1	1.64		3.9	
IV-1C	14.6	5.0	1.13		3.9		12.7	2.6	0.45		0.9	
IV-4A	11.3	5.1	1.45		2.9		12.2	6.6	1.05		5.6	
IV-4B							3.6	51.0	10.20		39.3	
IV-4C	11.6	18.2	7.82		12.8		11.3	14.4	4.63		9.8	
IV-4fl							13.0	2.1	0.42		2.0	
V	13.7	1.3	0.26		0.7		14.7	0.4	0.00		0.2	
Control Run 34							Turpentine Run 35					
Serum	12.4	5.2	1.6	19	2.1	65	12.1	8.3	3.1	31	4.1	77
II + III	14.0	3.7	1.2	25	2.2	80	12.3	13.5	5.3	29	4.7	70
II-3	15.4	0.9	0.2	16	0.2	48	13.9	3.1	1.4	13	1.1	80
II-1,2	14.8	0.3	0.0		0.1	25	14.9	0.3	0.0		0.2	35
III-1	14.8	2.1	0.4	29	0.8	46	13.9	5.3	2.8	9	0.5	74
III-0	13.7	6.1	2.0	28	2.1	62	12.0	11.8	4.9	34	3.7	69
IV-1	12.4	11.0	3.9	15	6.3	94	12.0	11.5	4.5	27	3.1	65
IV-4	12.6	9.4	4.1	16	6.4	100	11.7	12.8	4.9	34	5.3	71
IV-4B							7.2	34.4	1.1	64	31.3	63
IV-4C	11.9	8.9	5.7	14	5.2	100	11.7	11.0	5.4	23	7.8	100
IV-4fl							11.6	11.6	1.7	38	8.5	85
V	13.8	2.7	1.3	12	1.7	100	14.0	1.0	0.3	38	0.5	63

\* Cholesterol C + phospholipide C + ester cholesterol fatty acids C (gm per cent)

creased in Fraction V. The percentage of free cholesterol increased in Fractions II + III and IV-4 but decreased in Fraction V.

*Choline*—In order to characterize the type of phospholipide associated with various protein fractions, choline and phosphorus of the lipid extracts were determined for some lipid-rich fractions and the results are shown in Table X. Since the molar ratios of choline to phosphorus are all about unity, the phospholipide bound to protein is probably lecithin. Turog, Entenman, and Chaikoff (11) noted a similar relationship between choline and P for dog serum.

TABLE IX

*Nitrogen and Lipide Analyses of Protein Fractions in Control and Mustard-Treated Dogs*

Fraction No	Treatment	Nitrogen	Lipide C	Total cholesterol	Free cholesterol	Phospho lipid	C of lipid constituents accounted for
		gm per cent	gm per cent	gm per cent	per cent	gm per cent	per cent
II + III	M	13.0	5.6	2.1	40	2.0	67
	C	14.0	3.7	1.2	25	2.2	80
IV-1	M	9.5	14.2	2.6	32	9.0	63
	C	12.6	9.4	4.1	16	6.4	100
V	M	13.1	0.7	0.1	0	0.0	27
	C	13.8	2.7	1.3	12	1.7	100

\*M, mustard treated, C, control

TABLE X

*Percentage Distribution of Choline in Lipides of Some Serum Protein Fractions*

Fraction No	Control Dog 8		Turpentine Dog 7	
	Choline	Molar ratio Choline Phosphorus	Choline	Molar ratio, Choline Phosphorus
	gm per cent		gm per cent	
II + III			0.53	0.97
III-0A			1.35	0.94
III-2			1.19	0.97
IV-1A			1.48	1.01
IV-1B	0.73	0.95	0.74	1.14
IV-4B	1.69	0.94	6.04	0.93
IV-4C	1.95	0.94	1.55	0.96

*Carbohydrates*—Values for hexosamine hydrochloride are available for all fractions but are not listed, the minimum and maximum values range between 1.2 and 2.8 per cent. It is not possible to correlate the hexosamine concentration with the type of plasma protein. The highest value of 2.8 per cent is recorded for the  $\alpha$ -globulin-rich Fraction IV-4fl.

An attempt was made to determine the mannose and galactose concentrations of protein fractions by the Sorensen and Haugaard (12) procedure.

This method does not appear to give quantitative results for mixtures of sugars and the results must be considered as approximations. Since this procedure requires a water-soluble protein, only a few fractions could be analyzed. The alcohol-ether extracts of the lipid-rich Fraction IV-4B give positive reactions with the orcinol reagent, which indicates that lipides may interfere with the Sorensen-Haugard procedure.

Three analyses which are considered satisfactory give the results shown in Table XI. The total carbohydrate concentration of Fraction IV-4fl, which includes hexosamine and sugars, is 6.5 per cent.

TABLE XI  
*Carbohydrate Content in Some Serum Protein Fractions*

Fraction No	Sugar	Type	Total lipid C
	<i>per cent</i>		<i>per cent</i>
V	1.2	Mannose-galactose (2:1)	0.4
II-3	1.0	Mannose	1.2
IV-4fl	3.7	Mannose-galactose (2:1)	2.1

#### DISCUSSION

The data for the fractionation of the serum proteins of control and injured dogs are limited by the difficulties in obtaining sufficient amounts of sera. The presence of a comparatively large number of proteins in dog serum is indicated in these experiments. A great deal more work must be done to perfect the fractionation procedures with dog serum to isolate electrophoretically pure proteins.

The influence of injury on the protein-regenerating mechanism presents an interesting problem. An  $\alpha$ -globulin-rich fraction (No. IV-4B) containing large amounts of lipid has been found only in the serum of dogs injected subcutaneously with turpentine. A carbohydrate-rich fraction (No. IV-4fl) with the mobility of  $\alpha$ -globulin is present in the animals injured by turpentine and mustard. Two widely varying lipid concentrations are recorded for this fraction after turpentine injection. The lipid contents of these fractions differ in two groups and appear to depend on the condition of the animal and the state of the lipid metabolism. An  $\alpha$ -globulin-rich fraction termed Fraction IV is present in small quantities in the sera of most injured animals.

The available data show that there is a large difference in both the quantity and quality of the lipids associated with protein fractions as a result of injury. For example, not only does the total lipid C increase but the percentage of free cholesterol and phospholipids changes in the subfractions of Fraction II + III obtained from the sera of turpentine-



injected dogs These changes in the lipid constituents may indicate a redistribution of the lipid components as well as the introduction of new lipoproteins

### SUMMARY

Fractionation procedures are described for the separation of proteins of dog serum according to principles and methods developed by Cohn and associates

The percentage distributions of the proteins in main and subfractions are presented for the sera of control and injured (turpentine, heat, and bis( $\beta$ -chloroethyl) sulfide) dogs

Three  $\alpha$ -globulin-rich fractions, not present in detectable amounts in the serum of control dogs, can be separated from the serum of injured dogs

Analytical data for total lipid C, total cholesterol, free cholesterol, phospholipide, choline, hexosamine, and nitrogen are presented for many of the fractions

The  $\alpha$ - and  $\beta$ -globulins are associated with the increased lipid concentrations noted in the sera of injured animals

The authors are indebted to Mr Curtis S Floyd, Mrs Paula S Gjessing, Miss Frances E Haigh, Miss Lucy F Hoblitzel, Miss Florence L Jones, and Mr Robert F Mitchell for technical assistance

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# THE IMMUNOCHEMISTRY OF TOXINS AND TOXOIDS

## III THE ISOLATION AND CHARACTERIZATION OF DIPHTHERIAL TOXOID\*

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(Received for publication, June 14, 1947)

Crude diphtherial toxoid consists of formalin-treated diphtherial toxin and includes culture media constituents and bacterial products. These latter substances may elicit severe reactions in human beings. Thus, the purification of diphtherial toxoid is important for its use as an improved prophylactic agent against diphtheria and for the elucidation of its physical characteristics and chemical composition.

Although diphtherial toxin has been prepared in a highly pure state by Eaton (1) and by Pappenheimer (2), many attempts to purify diphtherial toxoid have met with varying degrees of success. Leonard and Holm (3) thoroughly reviewed the literature prior to 1933 and repeated the most promising purification procedures. They found the existing methods unsatisfactory. Methods, which gave high yields, produced a final product of a low degree of purity. Most of the purified toxoids showed signs of denaturation as indicated by either the absence or retardation of flocculation. It is of particular interest that alcohol or combined acid-alcohol precipitations in their hands proved unsatisfactory. Furthermore, these investigators experienced difficulty in sterilizing their products by filtration. Pope and Linggood (4), employing a complex lengthy procedure involving ultrafiltration, washing, adsorption, and elution, prepared a toxoid of about 70 per cent purity. Later, Linggood (5) modified Eaton and Gronau's (6) method in which cadmium chloride was used and again obtained a toxoid of about 70 per cent purity. These authors presented no data on the flocculation time or antigenicity of their products and thus the extent of denaturation or the antigenic character of the toxoids is unknown. Boivin (7), employing trichloroacetic acid precipitation, claimed a final product of about 2000 Lf per mg. of nitrogen. However, Pope and Linggood (4) were unable to confirm Boivin's results.

The practical and theoretical importance of preparing a stable purified diphtherial toxoid warrants further attempts. This is especially indicated in view of the success encountered in the crystallization of tetanal toxin.

\* Aided by a grant from the Lederle Laboratories Division, American Cyanamid Company.

(8) and the preparation of purified tetanal toxoid for clinical use (9, 10). A variable multiphase system of fractionation involving the precise adjustment of methanol concentration, pH, ionic strength, and protein concentration under controlled conditions of temperature has proved adaptable for the purification of many labile proteins (8-13). Cohn and associates (14, 15) employed a similar system in which ethanol instead of methanol was used for the purification of plasma proteins. Furthermore, it has been recognized here (10) that the addition of adjuvants, especially dipole ions such as glycine, to purified toxoids allows effective sterilization of the toxoid by filtration and markedly stabilizes the antigenic properties of the toxoid principle.

Accordingly, the present report concerns the isolation and characterization of diphtherial toxoid. Subsequent papers will deal with the preparation of the toxoids in a state suitable for human immunization.

### Materials

The crude diphtherial toxoids<sup>1</sup> employed in this study were produced from toxin prepared on a deferrated semisynthetic medium by use of a strain of *Corynebacterium diphtheriae* especially cultivated for this medium. The toxoid was formed by the addition of 0.5 per cent formalin to the toxin and aged until the material was non-toxic.

All reagents were either C. P. or the best grade obtainable. The acetate buffer for the pH adjustment of the toxoids is composed of 2.0 M acetic acid and 0.4 M sodium acetate. Diluted 40-fold with distilled water, this buffer gives a pH of 4.

### General Methods

The combining power of the toxoids was determined by the Ramon flocculation test. Increasing amounts of toxoid were added to a constant amount of antitoxin and the final volume brought to 2 ml with M/15 phosphate buffer of pH 7.4. After the contents of the tubes were mixed, incubation in a water bath at 50° was carried out until flocculation occurred. The tubes were read by transmitted light against a dull black background. The first tube showing flocculation was the indicator tube, and the time in minutes required for this flocculation was the Kf. Since a constant amount of antitoxin was employed, the Kf of different samples was comparable.

Methods for the electrophoretic analysis (16) and the ultracentrifugal studies<sup>2</sup> (17) have been reported elsewhere. The buffer used for electrophoresis had a pH of 8.6 and an ionic strength of 0.1. Solubility de-

<sup>1</sup> Supplied by Lederle Laboratories.

<sup>2</sup> We are indebted to Dr. J. L. Oncley for the ultracentrifugal analysis.

terminations were carried out as advocated by Northrop (18). Isoelectric points were determined by a modification of the method of Michaelis and Roni (19). Hydrogen ion determinations were made on the glass electrode. All pH determinations were made prior to the addition of alcohol after warming the sample to 25°. The ionic strength at the pH of the fractionations was calculated from the valence of the ions present and their concentration after alcohol addition. All centrifugations were carried out in a refrigerated centrifuge (International, PR-1).

Nitrogen was determined by the micro-Kjeldahl method of Pregl. Sulfur was determined by the lead acetate method, the nitroprusside reaction, and a modification<sup>3</sup> of a turbidimetric procedure (20). The highly sensitive test described by Feigl (21) was also used to detect sulfur. Iron and cobalt analyses were conducted spectriographically, and the sensitive  $\alpha, \alpha'$ -dipyridyl reaction (21) was also employed for the detection of iron. The usual qualitative tests for proteins, amino acids, phosphorus, and carbohydrate were carried out.

#### EXPERIMENTAL

*Theoretical Considerations*—The theoretical considerations of the precipitation of proteins by ethanol-water mixtures have been amply reviewed by Cohn and Edsall (22). Cohn and associates (14, 15) have subjected these theories to actual practice in the fractionation of the plasma proteins.

In studies on the purification of labile toxins and toxoids (8-13) methanol has been employed as the organic precipitating agent. It has been noted here that methanol leads to less irreversible changes in these labile proteins than does ethanol. Its relatively high dielectric constant and close chemical resemblance to water may determine its favorable properties. Since the volatile alcohol is removed from the proteins by freezing the mixture and then removing both the water and alcohol under reduced pressure, clinical objections to the use of methanol are eliminated.

It has been observed that the charged condition of the proteins determined by the pH of the mixture and the methanol concentration play the major rôle in this separation of toxins and toxoids. The temperature, by necessity, is maintained near the freezing point of the mixtures to avoid denaturation. Protein concentration is adjusted to allow protective stabilization of the toxoid molecules by virtue of protein-protein interactions and of their dipole activities. Within the narrow limits employed here (0.001 to 0.2), the ionic strength of the mixtures did not exhibit a solvent action but in the higher limits actually had a salting-out effect.

<sup>3</sup> The samples were oxidized in micro-Kjeldahl flasks with concentrated  $\text{HNO}_3$  and a few drops of bromine water. Turbidity measurements were made on a Klett photoelectric colorimeter.

The desired protein may be separated as a precipitate or may be maintained in solution, depending on the solubility limits of the protein and the impurities, the nature of the impurities, and the convenience of experimental conditions. A balance of five independent variables allows a large number of experimental conditions. It is the custom here to maintain four variables constant, concomitant with the variation of one condition. Thus, the solubility of the protein as a function of either pH, methanol concentration, protein concentration, or ionic strength can be determined as long as three of the conditions are kept constant.

The conditions finally established for each separation were those which resulted in the highest yield and greatest purity of the toxoid principle accompanied by no deleterious changes in its structure or function.

### *Results*

#### *Initial Separation of Toxoid Principle from Culture Medium*

In a series of solubility studies, the conditions for the separation of the toxoid principle from culture medium products were determined. The general fractionation method follows:

1 volume of toxoid is chilled to  $1^{\circ}$  and adjusted to the desired pH with ice-cold acetate buffer or, in some instances, with acetic acid. To this mixture, the calculated amount of methanol (measured at  $-5^{\circ}$  and chilled to  $-20^{\circ}$ ) is added slowly with constant stirring, care being taken to maintain the temperature at  $-5^{\circ}$  or under. The mixture is maintained at  $-5^{\circ}$  for 24 hours. At alcohol concentrations above 40 per cent, the temperature is maintained at  $-10^{\circ}$ , while at alcohol concentrations of 40 per cent or under, the temperature is kept near the freezing point or at  $-5^{\circ}$ . The precipitate is removed in a refrigerated centrifuge at the same temperature as that used during processing, and washed once with a methanol buffer-water mixture of the same composition as that employed for precipitation. The precipitate is then dissolved to one-tenth to one twentieth the original parent toxoid volume with  $1/15$  phosphate buffer of pH 7.4. The solution is clarified by centrifugation at 4000 RPM for 20 minutes at  $1^{\circ}$ , and then immediately assayed or stored at  $-25^{\circ}$  until needed.

The pertinent data on the behavior of the toxoid principle in mixtures consisting of culture media, methanol, and water under varying conditions of pH, ionic strength, and temperature are summarized in Tables I and II and other salient points portrayed graphically in Figs 1 and 2. Analysis of these results discloses the following information:

Diphtherial toxoid is quantitatively precipitated between pH 4 and 9 in 40 per cent methanol at ionic strength 0.09 and  $-5^{\circ}$ . The highest

TABLE I

*Precipitation of Diphtherial Toxoid in 40 Per Cent Methanol of Varying pH, Ionic Strength 0.09, Temperature -5°*

pH	N per ml	Lf per ml	Kf*	Lf per mg N	Yield†	Purification factor‡
	mg				per cent	
4.00	0.662	300	35	453	100	42
4.35	0.589	300	35	509	100	48
4.55	0.492	300	35	610	100	57
4.75	0.454	300	35	661	100	62
4.80	0.385	299	25	777	100	73
5.00	1.04	885	15	850	100	79
5.00	1.18	1020	30	865	100	81
5.15	0.332	292	25	879	90	82
5.30	0.239	233	25	975	71	91
5.40	0.230	206	25	862	64	81
5.45	0.234	200	30	855	61	80
5.60	0.207	140	30	676	42	63
5.85	0.130	58	40	446	18	42
Parent toxoid	3.15	34	40	10.7		

\* Incubation time, in minutes, determined at 35 Lf units

† Yield per cent =  $\frac{\text{total Lf of precipitate under indicated conditions}}{\text{total Lf in crude toxoid}} \times 100$

‡ Purification =  $\frac{\text{Lf per mg N in material precipitated under indicated conditions}}{\text{Lf per mg N in crude toxoid}}$

TABLE II

*Precipitation of Diphtherial Toxoid under Varying Conditions of Methanol Concentration, pH, Ionic Strength, and Temperature\**

Conditions				N per ml	Lf per ml	Kf	Lf per mg N	Yield	Purification factor
Methanol	pH	Ionic strength	Temperature						
per cent			C	mg				per cent	
0	4.05	0.15	0	0.272	175	45	643	57	60
0	4.60	0.15	0	0.184	58	55	315	19	29
10	4.00	0.13	-3	0.496	269	30	542	88	51
10	4.50	0.13	-3	0.423	219	30	518	71	48
10	5.10	0.13	-3	0.145	39	55	269	12	25
20	4.90	0.07	-5	0.190	148	90	779	91	73
25	4.00	0.11	-5	0.631	254	30	402	83	38
25	4.50	0.11	-5	0.498	280	30	562	90	52
25	5.10	0.11	-5	0.285	254	30	891	78	83
25	5.60	0.11	-5	0.147	50	55	340	15	32
60	5.40	0.11	-10	0.219	135	35	616	84	58
60	5.50	0.05	-10	0.242	152	30	628	95	59
Parent toxoid				3.15	34	40	10.7		

\* See foot-notes to Table I

concentration per mg of precipitated nitrogen is obtained between pH 4.9 and 5.3. Hydrogen ion concentrations greater than pH 5 lead to the increased precipitation of constituents other than toxoid. Hydrogen ion concentrations less than pH 5 result in an increased solubility of the toxoid principle. Increasing or decreasing the ionic strength under the above conditions does not appreciably affect the yield or purity. Elevation of the temperature to  $0^{\circ}$  in 40 per cent methanol generally results in de

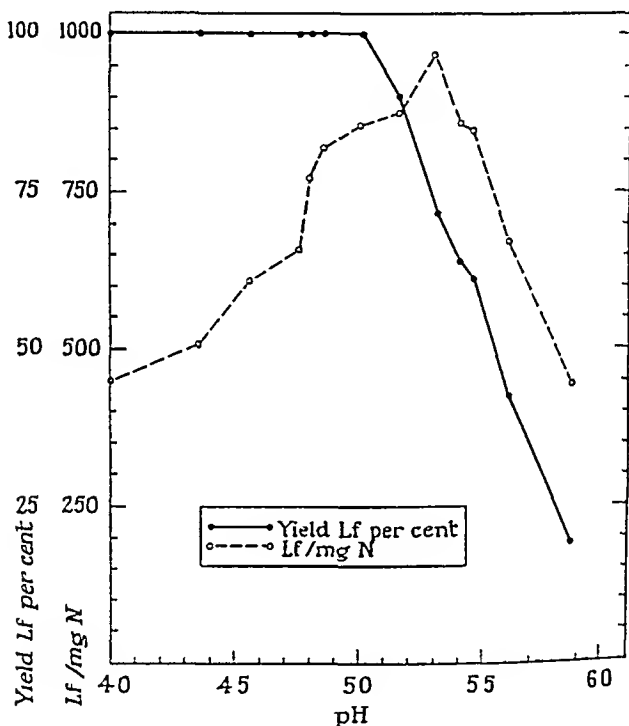


FIG. 1 Solubility of diphtherial toxoid in 40 per cent methanol at ionic strength 0.09 and at a temperature of  $-5^{\circ}$  as a function of pH

naturation of the toxoid.<sup>4</sup> Alcohol concentrations less than 40 per cent generally result in increased solubility of the toxoid. Alcohol concentrations greater than 40 per cent precipitate inert culture medium products and bacterial proteins. It was also noticed that some of the precipitates formed by lower alcohol concentrations had a slow Kf, indicating either damage to the toxoid *per se* or, more likely, separation of toxoid molecules which have been altered during the process of detoxification. There is no evidence that hydrogen ion concentrations between 4 and 7 under the

<sup>4</sup> Such toxoids show either an absence or marked retardation of flocculation



present conditions impair the toxoid principle, since a uniform Kf, often shorter than that of the parent toxoid, is observed over a wide pH range. The porphyrins or porphyrin-protein complexes are precipitated with the toxoid under pH 5.6.

The above information indicates that the separation of the toxoid principle from the crude toxoid occurs optimally at pH 4.9 to 5 in 40 per cent methanol at  $-5^{\circ}$ . The changed condition of the toxoid has a greater

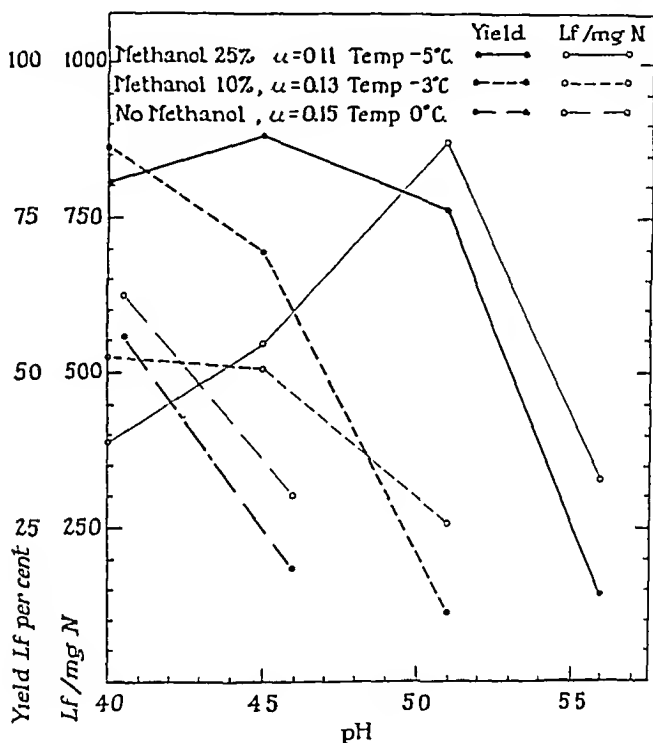


FIG 2 Solubility of diphtherial toxoid in different mixtures varying in pH, ionic strength, and temperature

influence on its solubility than the ionic strength of the mixture. The separation of bacterial toxins and toxoids studied here differs in this respect from the separation of the plasma proteins in ethanol-water mixtures, which depends largely on precise salt concentrations (15).

Note should be taken that a single precipitation quantitatively separates toxoid containing nearly 900 Lf per mg of nitrogen. Assuming that pure toxoid has 2200 Lf per mg of nitrogen (2), this material is 40 per cent pure. This precipitate is hereafter referred to as Fraction PI.

*Solubility of Toxoid Principle in Fraction PI*

Fraction PI was dissolved to one-tenth the parent toxoid volume in 0.15 M sodium acetate. The behavior of this solution in methanol-water mixtures was observed over a wide range of pH, ionic strength, and temperature. The results of these studies are summarized in Table III.

TABLE III  
*Precipitation of Diphtherial Toxoid in Fraction PI\**

pH	MeOH	Ionic strength	N per ml	Lf per ml	Kf	Lf per mg N	Yield	Purification factor
	per cent		mg				per cent	
4.0	5	0.075	0.088	15	150	170	10	16
4.0	15	0.075	0.101	68	95	742	44	69
4.0	30	0.05	0.150	155	95	1033	100	97
4.5	10	0.075	0.151	121	65	801	78	75
4.5	20	0.075	0.169	155	40	917	100	86
5.0	10	0.075	0.131	126	55	961	81	90
5.0	20	0.11	0.154	155	45	1007	100	94
5.3	10	0.12	0.117	126	65	1076	81	101
5.3	20	0.11	0.141	155	45	1099	100	103
5.7	25	0.075	0.101	121	55	1203	78	113
5.7	25	0.037	0.113	113	65	1000	73	93
5.7	30	0.20	0.104	142	58	1365	91	128
5.7	30	0.10	0.113	155	75	1371	100	128
5.7	40	0.09	0.132	155	35	1174	100	110
5.7	40	0.05	0.108	130	55	1204	83	112
5.7	40	0.025	0.091	121	55	1330	78	124
5.9	40	0.17	0.128	155	42	1203	100	112
5.9	40	0.086	0.111	155	58	1396	100	130
6.5	40	0.17	0.075	100	55	1333	64	124
6.5	40	0.09	0.058	68	55	1172	44	110
7.4	40	0.17	0.030	34	58	1133	22	106

\* See foot-notes to Table I

The toxoid principle in Fraction PI is quantitatively precipitated between pH 4 and 5.9 under appropriate conditions. It will be noted that initially 40 per cent methanol was necessary at any pH to precipitate the toxoid principle quantitatively and selectively. In Fraction PI, however, the toxoid is insoluble in 30 per cent methanol at pH 4.0, in 20 per cent methanol between pH 4.5 and 5.3, in 30 per cent methanol from pH 5.4 to 5.7, and in 40 per cent methanol at pH 5.9. Apparently, the removal of culture medium substances (proteins and protein degradation products) which may interact with the toxoid reduces the amount of

methanol required to precipitate the toxoid principle. Indeed, highly purified toxoid is largely precipitated at pH 4.7 to 4.9 in the absence of methanol. Between pH 4 and 5.3, varying the ionic strength between 0.05 and 0.20 did not greatly influence the solubility of either the toxoid or the impurities. However, it will be seen in Table III that an ionic strength of at least 0.09 is necessary for the quantitative recovery of toxoid accompanied by a high degree of purity at hydrogen ion concentrations less than 5.3. An effect similar to salting-out is observed here.

In view of the above considerations, reprecipitation of Fraction I at either pH 5.7 in 30 per cent methanol or at pH 5.9 in 40 per cent methanol at ionic strength of 0.09 and a temperature of  $-5^{\circ}$  is optimal for the quantitative recovery of the toxoid in the highest purity. These fractions have an N content of over 1300 per mg. of nitrogen.

It is of special importance to note that at pH 4 low methanol concentrations precipitate only about 10 per cent of the toxoid principle, while fully 50 per cent of the nitrogen is insoluble under these conditions. This observation is the basis for another step in the isolation of diphtherial toxoid.

### *Isolation of Diphtherial Toxoid*

Several large lots of crude diphtherial toxoid have been processed. The method given below has been found reproducible and lends itself well to large scale production.

In a preliminary note (13), two methods were given for the purification of diphtherial toxoid. The first step in these processes was identical and has been described above. However, in Method I, which has now been discarded, the porphyrins and the substantial quantity of bacterial proteins were removed by adsorption on asbestos in the presence of glycine. This procedure was lengthy and inconvenient, since it involved subsequent dialysis and multiple dryings from the frozen state. The method given below eliminates these objections and is employed routinely. However, work is in progress to improve even this procedure.

### *Purification of Diphtherial Toxoid<sup>5</sup>*

In Step I pH 4.9, 40 per cent methanol, ionic strength 0.09, temperature  $-5^{\circ}$  were employed. 1 volume of diphtherial toxoid previously chilled to  $1^{\circ}$  is adjusted to pH 4.9  $\pm$  0.05 with acetate buffer of pH 4 and ionic

<sup>5</sup> Diphtherial toxin is irreversibly inactivated by the above procedures. The toxin is extremely sensitive to hydrogen ion concentrations greater than pH 6.0 (1, 2). However, the combination of formaldehyde with the toxin results in a toxoid protein which is stable under the above conditions. Work is in progress on the nature and mechanism of this stabilization.

strength 0.4 The mixture is chilled to  $-5^{\circ}$  and the calculated amount of methanol (measured at  $-5^{\circ}$  and chilled to  $-20^{\circ}$ ) is added with stirring to a final concentration of 40 per cent methanol. The temperature is maintained at  $-5^{\circ}$ . After standing for 24 hours, the precipitate is collected at  $-5^{\circ}$ , dissolved to one-tenth of the original parent toxoid volume with 0.15  $\text{N}$  sodium acetate at  $0^{\circ}$ , and labeled Fraction PI.

In *Step II* pH 5.8, 40 per cent methanol, ionic strength 0.09, temperature  $-5^{\circ}$  were employed. Fraction PI is adjusted to  $\text{pH } 5.8 \pm 0.05$  with acetic acid and chilled to  $-5^{\circ}$ . Methanol (measured at  $-5^{\circ}$  and chilled to  $-20^{\circ}$ ) is added to Fraction PI to a final concentration of 40 per cent. The temperature is maintained at  $-5^{\circ}$ . The mixture is allowed to stand for 24 hours, after which the precipitate is collected at  $-5^{\circ}$ , dissolved to one-fortieth the original parent toxoid volume with 0.15  $\text{N}$  sodium acetate at  $-3^{\circ}$ , and labeled Fraction PII.

In *Step III* the conditions were pH 4.0, 5 per cent methanol, ionic strength 0.05, temperature  $-3^{\circ}$ . Fraction PII is rapidly adjusted with stirring to pH 3.95 to 4.0 with acetic acid at  $-2^{\circ}$ . 2 volumes of 7.5 per cent methanol ( $-3^{\circ}$ ) are then added and the mixture is stirred for 2 hours at  $-2^{\circ}$ . The precipitate is then collected at  $-2^{\circ}$  and discarded. The supernatant is stored at  $-2^{\circ}$  and labeled Fraction SIII.

In *Step IV* pH 4.4, 30 per cent methanol, ionic strength 0.06, temperature  $-5^{\circ}$  were employed. Fraction SIII is carefully adjusted to  $\text{pH } 4.4$  with NaOH at  $-3^{\circ}$ . The mixture is chilled to  $-5^{\circ}$  and sufficient methanol previously chilled to  $-20^{\circ}$  is added to bring the final concentration to 30 per cent. The temperature is maintained at  $-5^{\circ}$  or lower. The mixture is allowed to stand at  $-5^{\circ}$  for 48 hours. The precipitate is collected at  $-5^{\circ}$  and dissolved up to one-twentieth of the original parent toxoid volume with 0.15  $\text{N}$  sodium acetate at  $-2^{\circ}$  and labeled Fraction PIV.

In *Step V* the conditions were pH 5.4, 25 per cent methanol, ionic strength 0.06, temperature  $-5^{\circ}$ . Fraction PIV is adjusted to pH 5.4 with sodium acetate and NaOH so that the final ionic strength after the addition of the methanol will be 0.06. The mixture is then chilled to  $-5^{\circ}$  and sufficient methanol is added to bring the final concentration to 25 per cent. The temperature is maintained at  $-5^{\circ}$  or lower. After standing for 48 hours, the precipitate is collected at  $-5^{\circ}$  and dissolved up to one-fortieth of the original parent toxoid volume at  $0^{\circ}$  with either sodium acetate, phosphate buffers, or glycine, depending on its intended use. This material is then dried from the frozen state to less than 1 per cent moisture. The dried toxoid may be stored indefinitely or may be redissolved for

\* pH determined on an aliquot of Fraction SIII diluted 1:2 with water.

assay or analysis. If the material is to be sterilized by filtration, it must be dried in the presence of certain dipolar ions, preferably glycine (10).

The above procedure has generally resulted in a toxoid free of bacterial proteins and porphyrin. However, if adequate purification is not achieved, one or more of the above steps may be repeated. The yield of purified toxoid has been about 55 to 80 per cent in the fractionations carried out here. The purification and yield achieved at each step are given in Table IV.

The first two steps in this procedure substantially separate the toxoid principle from culture medium and bacterial products. Nevertheless, fully 30 per cent of Fraction PII is composed of bacterial protein and

TABLE IV  
*Degree of Purification and Yield of Diphtherial Toxoid during Fractionation*

Fraction	Lf per mg N (average values)	Kf (at 50 Lf units)	Yield of parent toxoid
			<i>per cent</i>
PI	900	35-55	90-100
PII	1300	25-40	90-100
SIH	1800	25-40	65-85
PIV	2000	10-20	60-85
PV	2100-2200	10-20	55-80
Parent toxoid	10-15	40-65	

porphyrin complexes. These substances were removed formerly by adsorption on asbestos fibers in the presence of dipolar ions. A comprehensive study of the solubility behavior of this fraction revealed that at pH 4 and at 5 per cent methanol, inert proteins were insoluble, while most of the toxoid principle remained in solution. The toxoid principle may then be further purified as indicated under Steps IV and V, resulting in a final product containing over 2100 Lf per mg of nitrogen.

It was noted that removal of the bacterial proteins, etc., at pH 4 also resulted in the precipitation of some toxoid. This material produced poor flocculation of antitoxin and had a Kf of over 6 hours. This may indicate that the toxoid molecules insoluble at pH 4 are chemically or structurally distinct from the toxoid soluble under these conditions. Detoxification of toxin by formalin may lead to a partial alteration in certain toxoid molecules, or the crude toxin *per se* may have contained incomplete or degraded molecules. If this hypothesis is substantiated, the removal of low grade toxoid at this step is desirable and should not be considered a true loss. In a subsequent publication it will be shown further that

purified toxoid elicits a greater antibody response than does a comparable dose of crude toxoid.<sup>7</sup> It is also well known that crude toxoid may contain substances which interfere with immune response. This interference may resemble the phenomena of analogue competition encountered in other fields.

### *Characterization of Purified Diphtherial Toxoid Fraction PV*

*General Properties*—Fraction PV is pale yellow in a 1 per cent solution in distilled water. Its chemical composition is summarized in Table V.

TABLE V  
*Chemical Composition of Purified Diphtherial Toxoid*

Constituents	Fraction PV
Nitrogen, %	16.6
Tyrosine	+++
Arginine (guanidine)	+++
Tryptophan	+
Carbohydrate	—
Phosphorus	—
Sulfur	—
Iron	—
Cobalt	—

TABLE VI  
*Properties of Purified Diphtherial Toxoid*

Mobility in veronal buffer, pH 8.6, 0.1 ionic strength	$8.1 \times 10^{-5}$
Sedimentation constant, $s_{20}^{1\%}$ , w	4.6
Optical rotation, $[\alpha]_D^{25}$ , degrees	-45
Isoelectric point	$4.7 \pm 0.1$
Lf per mg N	2190
Kf <sub>40</sub> , min	12
Solubility, 1.8 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , pH 7.4	Constant

While purified diphtherial toxoid gives the usual protein reactions, it is entirely lacking in phosphorus, sulfur, carbohydrate, iron, cobalt, or porphyrin.

*Physicochemical Properties*—Table VI summarizes the physicochemical character of Fraction PV. The toxoid is electrophoretically homogeneous.

<sup>7</sup> Kekwick and Record (23) have shown that the immunization of horses with crude diphtherial toxoid gives rise to two antitoxins. The  $\gamma$  fraction flocculates rapidly with toxin, while the  $\beta$  fraction flocculates slowly. Whether this phenomenon is related to the presence of two chemically different toxoids, as noticed here, still remains obscure. Work is in progress to clarify this possible relationship.

(Fig 3) with a mobility of  $8.1 \times 10^{-5}$  in veronal buffer of 0.1 ionic strength at pH 8.6. The molecules sediment with the same velocity, calculated to be 4.6 Svedberg units, in the ultracentrifuge. Inspection of Fig 4 reveals that Fraction PV has constant solubility in 1.8 M ammonium sulfate at

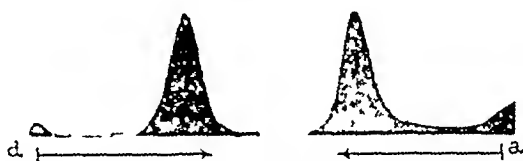


FIG 3 Electrophoretic diagrams for diphtherial toxoid, Fraction PV

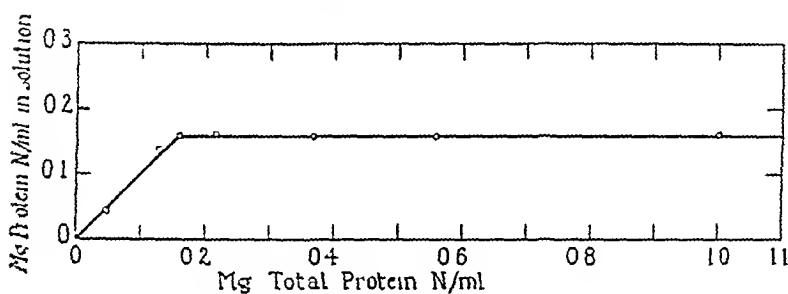


FIG 4 Solubility curve of diphtherial toxoid, Fraction PV

TABLE VII  
*Solubility of Fraction PV in Distilled Water As Function of pH*

pH*	Lf pptd	Protein N pptd
	<i>per cent</i>	<i>per cent</i>
3.65	0	
4.35	30	
4.7	90	90
4.9	80	80
5.1	50	50

\* Determined on supernatants after equilibration with the precipitates for 18 hours at 1°

pH 7.4 and acts as a solid phase of one component. The optical rotation of  $-45^\circ$  compares favorably with that reported for purified toxin (1, 2).

The isoelectric point was estimated from the minimum solubility of both the total nitrogen and the combining activity of Fraction PV. The purified toxoid was adjusted to different hydrogen ion concentrations with acetic acid at 0.2 pH intervals in distilled water at constant volume and temperature. The results of this experiment are given in Table VII. It

will be noted that, judged by both nitrogen and combining capacity, the purified toxoid has a minimum solubility at  $\text{pH } 4.7 \pm 0.1$

While no claim of absolute purity is made, Fraction PV satisfies the existing criteria of a pure protein

*Immunologic Evaluation*—Subsequent papers will deal with the immunological aspects of purified diphtherial toxoid. It will suffice here to state that the purified samples flocculate sharply and rapidly with antitoxin and are highly antigenic. Fraction PV is not precipitated by an anti-*Corynebacterium diphtheriae* rabbit serum. The addition of dipolar ions such as glycine allows satisfactory sterile filtration and greatly enhances the stability of the purified toxoids

#### SUMMARY

Methods for the separation of diphtherial toxoid in highly purified state with satisfactory yields are described. The methods consist of a multi-phase fractionation system involving methanol as the precipitating agent under controlled conditions of pH, ionic strength, protein concentration, and temperature

Diphtherial toxoid has been separated as a homogeneous, water-soluble, simple protein with an electrophoretic mobility of  $8.1 \times 10^{-5}$  in veronal buffer of 0.1 ionic strength at pH 8.6 and with a sedimentation constant of 4.6 Svedberg units. The preparation has constant solubility and satisfies the existing criteria for a pure protein. The purified toxoid contains no sulfur, phosphorus, carbohydrate, iron, cobalt, or porphyrin

The purified diphtherial toxoid does not precipitate anti-*Corynebacterium diphtheriae* rabbit serum. The final product contains between 2000 and 2200 Lf per mg of nitrogen. It produces sharp flocculation of antitoxin, with a marked decrease in the flocculation time compared with crude toxoid

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# THE RELATION OF THE DIET TO THE COMPOSITION OF TISSUE PHOSPHOLIPIDES

## VII EFFECTS OF LACTOSE-CONTAINING DIETS\*

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(Received for publication, July 10, 1947)

In the previous papers of this series (2, 3) the carbohydrate component (dextrin-sucrose 1:1) of the experimental diets was maintained constant, while the effect on liver lipides of various nutrients was being studied. Later, it seemed logical to study the effects of altering the nature of the dietary carbohydrate.

Preliminary results obtained in experiments in which lactose was the sole carbohydrate in the diets were reported in 1944 (4). In contrast to the controls on the low fat, sucrose-dextrin diet, which showed a marked fat infiltration of the liver, only moderate amounts of fats were found in the livers of the rats on the lactose-containing diets. The lecithin levels were also frequently not as low as in the controls. Moreover, the effectiveness of choline supplementation in reducing the fat infiltration and in raising the lecithin level in the liver appeared greater in the animals on the lactose diets. However, these rats exhibited profuse diarrhea, anorexia, marked loss in weight, and, although no deaths occurred during the experimental period, they looked definitely ill. Similar findings with high lactose diets have since been described by others (5-8).

It was found that by replacing only sucrose with lactose in the diet, the animals remained in good health. By this modification, the interpretation of the data was made somewhat simpler since the lipides were under the influence of one less variable (absence of dextrin). In the course of the study, the question arose whether or not lactose had lipotropic properties in animals in which the fatty liver was produced by a high fat rather than by a high carbohydrate ration.

In the present paper, data are presented concerning the effects on liver lipides of lactose-containing diets of variable fat content. The possible mechanisms by which the substitution of lactose for sucrose in the diet produces its effects are pointed out and discussed.

\* Aided by a grant from the John and Mary R. Markle Foundation. A preliminary report has appeared (1).

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## EXPERIMENTAL

Each experimental group consisted of three or four male albino rats raised on our stock diet to 100 to 110 gm of body weight. The composition of the experimental diets is recorded in Table I. The conditions of the various experiments duplicated those of the experiments reported in our previous papers (2, 3). That is (a) the animals were transferred from the stock diet directly to the sucrose- or lactose-containing diets and main-

TABLE I  
*Composition of Experimental Diets\**

The values are in per cent of dry weight

Diet No	Sucrose	Lactose†	Dextrin	Glucose	Maltose	Galactose	Crisco
4	37		37				5
8		37	37				5
11	27		27				25
10		27	27				25
17	54						25
9		54					25
18			54				25
13				54			25
14					54		25
16						54	25

\* In addition to the above components, all experimental diets contained casein (Labeo, vitamin-free) 10 per cent, cod liver oil 5 per cent, a salt mixture (Osborne and Mendel (9)) 4 per cent, and a cellulose material (Ruffey) 2 per cent. Pure B vitamins in the amounts previously indicated (3) and, in the experiments with choline supplementation, also choline hydrochloride (50 mg per rat) were incorporated in the daily rations.

† The lactose used in the preparation of Diets 8, 10, and 9 was U S P lactose (Merck). Diets 9a and 9b were identical to Diet 9 except that in Diet 9a this material has been submitted to four recrystallizations (two from water and two from 50 per cent ethyl alcohol) and in Diet 9b, lactose c p (Baker) was employed.

tained on these diets for 12 days ("12 day experiments"), or (b) the rats were first transferred for 7 days to one of the sucrose-dextrin diets, then maintained for 12 more days on either the same diet or the lactose-substituted diet ("7 + 12 day experiments"), or (c) and (d) the conditions of the experiments were similar to those described under (a) and (b) respectively, except that during the (last) 12 days choline hydrochloride (50 mg per rat per day) was added to the diets (Tables II and III). In addition, some preliminary experiments are included in which various carbohydrates were substituted for both the sucrose and dextrin in high fat diets (Table IV). Procedures and analytical methods have been described (2).

TABLE II

*Effects on Liver Lipides of Substituting Lactose for Sucrose in Experimental Diets*  
 Lipide values in mg per 1 gm of moist lipide-free tissue

Experiment No	No of rats and analyses	Diet No	Per cent fats in diet	Per cent lactose in diet	Daily food intake	Change in body weight	Liver weight for 125 gm rat	Total lipides	Phospholipides				Non phospholipide fatty acids	Unsatouifiable matter	
									Total	Choline- containing		Non choline containing			
										Per cent total phos- pholipides					
No choline added to diets															
1	4 (2)	4	10	0	12.4	+12.1	6.20	107.7	22.8	12.7	56	10.1	73.0	4.6	
2	4 (2)	8	10	37	9.2	+2.1	4.57	69.2	23.1	12.3	54	10.8	32.7	10.1	
3-4*	6 (4)	4	10	0	12.4	+19.3	5.74	141.8	22.7	11.7	52	11.2	100.6	8.4	
5*	4 (2)	8	10	37	9.4	+14.7	5.04	71.0	21.4	11.8	55	9.6	38.4	9.2	
6	4 (2)	11	30	0	8.2	+27.2	6.82	193.4	20.6	11.5	56	9.1	148.0	10.0	
7	4 (2)	10	30	27	7.3	-2.4	5.15	89.0	24.8	14.6	59	10.2	52.5	6.4	
8†	4 (2)	11	30	0	6.3	-0.8	6.70	320.4	17.4	11.1	64	6.3	259.6	17.8	
9†	4 (2)	10	30	27	5.3	+1.9	5.56	170.2	26.6	13.4	50	13.2	121.3	10.2	
10†	4 (2)	11†	30	0	5.6	+2.9	6.10	278.3	23.9	11.7	49	12.2	224.0	8.0	
11-12†	8 (4)	10†	30	27	5.6	-4.0	5.67	173.8	23.3	12.4	53	10.9	123.9	14.2	
Choline added to diets															
13	5 (2)	4	10	0	11.7	+26.6	5.79	65.5	23.6	16.4	70	7.2	30.6	8.2	
14-15	6 (4)	8	10	37	12.4	+17.7	4.79	40.5	28.7	18.4	64	10.3	7.4	3.7	
16-18*	14 (8)	4	10	0	10.2	+24.6	5.35	51.0	23.2	13.2	57	10.0	20.6	4.1	
19-20*	7 (4)	8	10	37	10.0	+24.0	5.80	38.3	20.5	14.6	72	5.9	11.1	5.1	
21*	4 (2)	8†	10	37	7.4	+7.0	5.27	33.8	19.7	13.4	68	6.3	8.8	4.4	
22	4 (2)	11	30	0	10.0	+40.0	5.52	63.8	23.2	17.3	75	5.9	29.7	7.9	
23	4 (2)	10	30	27	6.2	-3.3	5.50	44.6	26.0	21.9	84	4.1	9.7	7.9	
24†	4 (2)	11	30	0	7.4	+12.6	5.61	72.5	21.1	17.9	85	3.2	41.3	6.0	
25-26†	7 (4)	10	30	27	5.6	-0.5	5.38	79.3	26.8	24.0	90	2.8	40.9	7.5	
27†	4 (2)	11†	30	0	6.2	+14.0	5.94	141.0	25.4	20.8	82	4.6	98.5	7.2	
28-29†	8 (4)	10†	30	27	4.3	-5.8	5.16	72.8	23.6	20.0	85	3.6	37.5	7.9	

\* Prior to the experiments, rats of these groups were maintained for 7 days on Diet 4

† Prior to the experiments, rats of these groups were maintained for 7 days on Diet 11

‡ 2 per cent sulfasuxidine was added to the diets, and p aminobenzoic acid was omitted from the vitamin mixture

### Results

The rats on lactose-dextrin diets appeared to be in a good state of nutrition and did not exhibit marked abnormal symptoms. Occasionally some of them had a mild diarrhea and at autopsy a certain degree of distention of the gastrointestinal tract was noted. Usually these rats ate less food and grew less well than the rats on sucrose-dextrin diets but the differences were not constant and often not very marked, as shown in Table II. However, their livers were smaller. In Table II the complete

TABLE III

*Comparison of Changes in Liver Lipids Induced by Dietary Lactose and Choline*

The figures express the increase (+) or decrease (−) from the values obtained in the corresponding experiments on rats on sucrose dextrin diets (Table II)

Liver lipid fraction	Duration of experiment				
	12 days			7 + 12 days	
	Nutrient tested	Low fat (Diet 4 or 8)	High fat (Diet 10 or 11)	Low fat (Diet 4 or 8)	High fat (Diet 10 or 11) with sulfa suzidine
Lecithins					
Choline phospho lipides in 1 gm moist lipid free tissue	Lactose	−0.1	+3.1	+0.1	+2.3
	Choline	+3.7	+5.8	+1.5	+6.8
	Lactose + choline	+5.7	+10.5	+2.9	+12.9
Neutral fats					
Non-phospholipide fatty acids in 1 gm moist lipid free tissue	Lactose	−40.3	−95.5	−62.2	−138.3
	Choline	−12.4	−118.3	−80.0	−218.3
	Lactose + choline	−65.6	−138.3	−89.5	−218.7

data have been arranged so that the results of the control experiments on rats on the sucrose-dextrin diets may be compared directly with the values of the lactose-dextrin experiments recorded in the next line. The important differences observed have been summarized in Table III for the sake of convenience.

In the experiments without choline supplementation, the most striking effect of lactose substitution is a decrease in the values for the total lipides and especially for the non-phospholipide fatty acids. The decrease is apparent with both low and high fat diets in experiments of 12 and 19 days duration.

When choline was added to the diets, the lipotropic action of this substance was always marked. However, at least in most experiments, the livers of the animals on the choline-supplemented, lactose-substituted diets contained definitely lower amounts of total lipides and neutral fats. The regular occurrence of the lipotropic effects of lactose in many experiments of varying construction should be noted, in view of the frequent finding of large individual variations in liver fat which have been encountered in investigations of lipotropic factors.

TABLE IV

*Effects on Liver Lipids of Substituting Various Carbohydrates for Sucrose and Dextrin in Experimental Diets*

All diets contained 50 per cent fat and the rats were transferred from the stock diet to the experimental diets (immediate substitution). Lipide values in mg per 1 gm of moist lipide free tissue.

Experiment No	No of rats and analyses	Diet No	Type of carbohydrate in diet	Daily food intake		Change in body weight	Liver weight for 125 gm rat	Total lipides	Phospholipides					Non phospholipide fatty acids	Unassonifiable matter
									Total	Choline containing		Non choline containing			
										Per cent total phospholipides	phospholipides				
													mg		
				gm	gm	gm	mg	mg	mg	mg	mg	mg	mg	mg	
64 (2)	11	Sucrose dextrin		8.2	+27.2	6.82	193.4	20.6	11.5	56	9.1	148.0	10.0		
303 (2)	17	Sucrose		5.7	+0.7	7.17	172.5	22.6	12.1	54	10.5	128.8	8.2		
314 (2)	13	Glucose		8.5	+11.4	6.05	175.3	20.8	11.8	56.8	9.0	134.3	6.8		
324 (2)	14	Maltose		9.5	+19.5	5.75	195.1	24.5	15.2	62	9.3	146.2	9.8		
333 (2)	18	Dextrin		7.6	+9.3	7.96	88.1	25.1	15.6	62	9.5	52.4	5.4		
344 (2)	9	Lactose, U S P		5.8	-7.0	5.00	102.5	24.4	11.3	46	13.1	63.1	8.7		
354 (2)	9a	Lactose, U S P, recrystallized		5.2	-3.0	5.24	135.7	23.1	14.4	62	8.7	92.0	11.4		
364 (2)	9b	Lactose, c r		4.8	-4.8	4.86	84.4	23.0	14.1	62	8.9	50.5	5.8		
37-38	8 (4)	16 Galactose		6.9	-20.7	7.46	89.3	27.2	17.5	64	9.7	50.3	6.7		

In agreement with our previous observations (3), choline supplementation raises the liver lecithin level in the animals on low fat sucrose-dextrin diets if it is initiated immediately (12 day experiments), whereas only minimal changes are detectable when the supplementation is preceded by a 7 day period on the unsupplemented diet (7 + 12 day experiments). On the other hand, choline supplementation (both immediate and delayed) causes a marked increase in the level of liver lecithin in the high fat-sucrose-dextrin groups.

Substantially identical effects were observed with choline-supplemented,

lactose-containing diets, but, usually, the increase in liver lecithins was more considerable than in the corresponding experiments with sucrose-containing diets. The inclusion of sulfasuxidine in the diets did not alter the above findings.

From Table IV, in which various carbohydrates were substituted for both the sucrose and the dextrin in the high fat diets, these findings may be stated. The sucrose-, glucose-, and maltose-containing diets produced the same degree of liver fat infiltration as in the rats fed sucrose-dextrin diets. Lecithin values were low with the glucose- and sucrose-containing diets but rather elevated in the rats fed the maltose-containing diet. A relatively high lecithin level and only moderate amounts of fats were found in Experiment 33, in which dextrin represented the sole carbohydrate component of the diet. The results obtained in the three experiments with diets containing 54 per cent lactose were essentially the same, although lactose with various degrees of purity was used for each experiment. The results of these experiments almost duplicate those of Experiment 7 made under similar conditions except that a lactose-dextrin diet was employed. In the experiments with galactose-containing diets, the fatty infiltration was in the same range as in the rats on lactose-containing diets, but higher values were obtained from the lecithins in the liver. It should be pointed out that the number of animals studied (Table IV) was small and the data should be regarded as suggestive.

From a consideration of all the results, there appears to be a similarity in the effects of lactose substitution and of choline supplementation.

#### DISCUSSION

The evidence obtained in the present experiments, in which only part of the carbohydrate in the diet was represented by lactose, confirms and extends our preliminary findings on rats fed diets with a higher lactose content (1, 4). In both studies, the effects of lactose substitution resembled the action of choline by reducing liver fat and by elevating liver lecithin concentration. Low values for total fatty acids in the livers of rats on high lactose and high galactose diets (7) have also been observed more recently by others, in young animals this finding was accompanied by the absence of hemorrhagic lesions in the kidneys (8). It appears therefore that the partial or total substitution of lactose for the carbohydrate component in low protein diets causes a definite alleviation of the symptoms considered characteristic of choline deficiency.

The elucidation of the mechanisms by which the substitution of lactose for other carbohydrates in the diet produces its effects presents some difficulty. Obviously, an attenuation of the symptoms of choline de-



iciency may result from either an increased availability of or a decreased demand for choline for lipotropic purposes

It is possible that choline or its precursors may have been present as an impurity in the dietary lactose. However, this view is not favored by the results of the experiments in which lactose of different degrees of purification was employed in the preparation of the diet. Indeed, the highest content of liver fat was observed in the experiment in which the material was most carefully purified.

Choline may have been manufactured in excessive amounts either by the intestinal flora or by the animal tissues. Thus, considerable changes occur in the intestinal flora of animals on lactose-containing diets and it has been shown that these changes may lead to an increased formation of certain essential dietary factors (10). It is conceivable that the substitution of lactose for sucrose could enhance the synthesis of choline by microorganisms in the intestine (perhaps also, the synthesis of some unknown factor which might be involved in the formation of choline or in its utilization for the formation of liver phospholipides). The experiments in which sulfasuxidine was added to the diet were devised in order to test this hypothesis, but the results of these experiments are far from conclusive. Likewise, we do not have evidence for or against the idea that in the rats on lactose-containing diets more choline in animal tissues is formed by synthesis, or less of it is utilized for non-lipotropic purposes.

A decreased demand for choline could perhaps best explain the close relationship between the growth of the animals on low choline diets and the degree of fat infiltration in the liver, which has been recently emphasized (7, 11). Generally speaking, such a relationship may be found also in our experiments, since most rats on lactose-containing diets grew less than the controls on the sucrose-dextrin diets. It should be noted, however, that in several instances differences in the rate of growth between rats on the two types of diets and under the same experimental conditions were not apparent or were only very slight, whereas, even in these instances, the amounts of fat in the liver of the animals on the lactose-containing diets were consistently lower (see, for example, Experiments 3-4 and 5, 8 and 9, 16-18, and 19-20).

If the substitution of lactose in the diets does not make larger amounts of choline available for lipotropic purposes, the decrease in the fatty infiltration may be due to a diminished supply of fatty acids to the liver. An impaired absorption or utilization of carbohydrates, with a consequent decrease in the extent of their conversion to fats, seemed a very likely explanation for the results of our preliminary experiments in which low fat diets with a very high lactose content were employed. However, the

differences in the degree of fat infiltration caused by the lactose substitution are still quite marked in the experiments in which diets with high fat content were employed, that is, in a condition in which the formation of fats from carbohydrates is certainly not the main source for the fats accumulating in the liver. Of course, in these experiments the existence of differences in the amounts of fats ingested or absorbed should still be considered.

In conclusion, it is apparent that none of the available evidence is sufficient for accepting or rejecting any of the interpretations which we have discussed.

As for the few experiments in which high fat diets containing various carbohydrates were employed, our findings suggest that the effects of lactose substitution are not entirely specific for this carbohydrate, since results more or less similar to those obtained in the experiments with the lactose-substituted diet have also been observed with diets containing other carbohydrates<sup>1</sup>. On the basis of the results of the experiments with galactose-containing diets, it seems not unlikely that the effects of the substitution of lactose may be chiefly ascribed to the galactose moiety of its molecule.

#### SUMMARY

The lipide composition of the liver of rats maintained on low protein diets, in which the carbohydrate component consisted of equal parts of dextrin and sucrose, has been compared with that of the liver of rats fed similar diets except that lactose was substituted for sucrose.

Smaller amounts of neutral fat were found in the livers from animals on lactose-containing diets than those of the rats on the unsubstituted diets. The effectiveness of choline supplementation in reducing the fat infiltration and in raising, under certain experimental conditions, the lecithin level in the liver appears greater in animals maintained on the lactose-containing diets.

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<sup>1</sup> It should be mentioned that the substitution of carbohydrates, other than lactose, for the sucrose component of synthetic diets may also prevent the appearance of hemorrhagic kidneys in very young rats. Thus, starch (12), galactose (8), perhaps also glucose (8), have been found to be more or less effective.

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# DETERMINATION OF MICRO QUANTITIES OF CITRIC ACID IN BIOLOGICAL FLUIDS

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(Received for publication, July 1, 1947)

The importance of citric acid in carbohydrate metabolism has become increasingly apparent in recent years (1). However, the significance of citric levels in human blood in health and disease has been handicapped by the lack of suitable method for this determination in small quantities of blood. Krog (2) reports a method for the determination of citric acid in 3 ml. of blood by converting it to acetone, distilling the acetone, and allowing the distillate to react with salicylaldehyde. The procedure is too elaborate for routine determination.

Examination of the literature indicates that two methods have been generally used in experimental work by which amounts of the order of 100  $\gamma$  could be determined with any degree of accuracy. Determination of this amount would correspond to the amount in 7 to 10 ml. of whole blood. Obviously if duplicates and recoveries were run, the amount taken for one determination would be in excess of 20 ml. of blood and would therefore be impractical for routine studies.<sup>1</sup>

The two procedures referred to are the Thunberg method (5-11) and the pentabromoacetone method as developed by several investigators (3, 12-19). Thunberg's method is an enzymatic determination, which is subject to many interfering substances, requires elaborate technique, and is time-consuming, making it unsuitable for clinical determinations on large numbers of bloods. Two variations of the second procedure have been reported, both of which make use of the observation of Cahours (20) that citric acid yields pentabromoacetone on bromination. The pentabromoacetone is then determined either by its colorimetric reaction with a solution of sodium sulfide (17) or by determination of bromine after the decomposition of the pentabromoacetone with sodium sulfide (16, 18, 19, 21). Pucher's colorimetric variation (17) was chosen as lending itself to refinement to micro quantities.

In the colorimetric method described by Pucher the pentabromoacetone is extracted with petroleum ether, the petroleum ether is removed in a

<sup>1</sup> Since this paper was written a method for the determination of citric acid, after the method of Kometiani (3) for amounts from 10 to 60  $\gamma$  has been published (4).

separatory funnel, washed, transferred to a second separatory funnel, and the color is developed by adding sodium sulfide solution. The color, now in the sodium sulfide solution, is separated, the petroleum ether is washed, and the washings combined. Pyridine is added to stabilize the color and the solution is made up to 10 ml and the color read in a Pulfrich spectrophotometer with a No 430  $m\mu$  filter. In this volume the color is not intense enough to be used for microanalysis.

A study was first made of the absorption spectrum of this color. As can be seen from Fig 1, the maximum absorption is at 450  $m\mu$  and not at 430  $m\mu$  as recommended by Pucher or at 470  $m\mu$  as recommended by

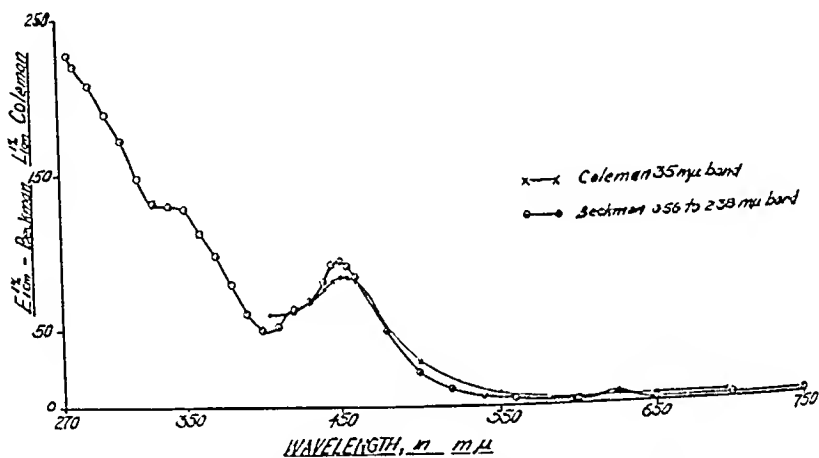


FIG 1 Absorption spectrum on Beckman and Coleman spectrophotometers of color produced by the action of 4 per cent sodium sulfide solution on pentabromo acetone

Josephson and Forssberg (22) or at 420  $m\mu$  as recommended by Hunter and Leloir (23). None of these investigators reported the absorption curve. Approximately 10 per cent increase in sensitivity can be obtained by using this wave-length. It is to be noted that the extinction coefficient is only  $E_{1\text{ cm}}^{1\%} = 92$ . This indicates that for micro application the color would have to be developed in small volumes to work down to a method suitable for 1 ml of blood.

Pucher and other investigators in this field read the color against water as a standard. In actual practice we find that the sodium sulfide solution itself when read against water never shows 100 per cent transmission but varies from 89 to 99 per cent. It is apparent that this would cause the results to vary widely with dilute solutions. In this investigation the colors are read against the actual sodium sulfide solution used. The

sodium sulfide solutions are checked beforehand and any solution reading less than 95 per cent transmission against water is rejected

The addition of pyridine as recommended by Pucher to stabilize the color is found to be unnecessary Josephson and Foissberg (22) note that results by the Pucher method do not strictly follow Beer's law and vary with the sample of pyridine They therefore substitute redistilled glycerol to stabilize the color Hunter and Leloir recommend the addition of

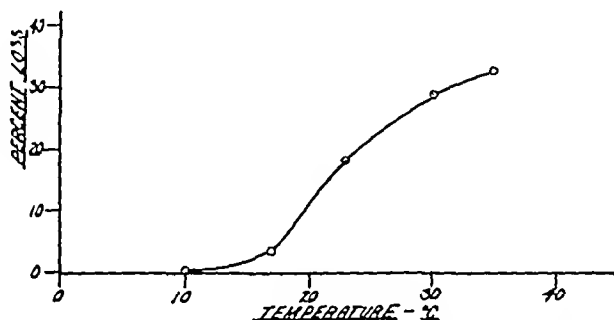


FIG 2 Effect of temperature on the percentage color loss (time constant, 35 minutes)

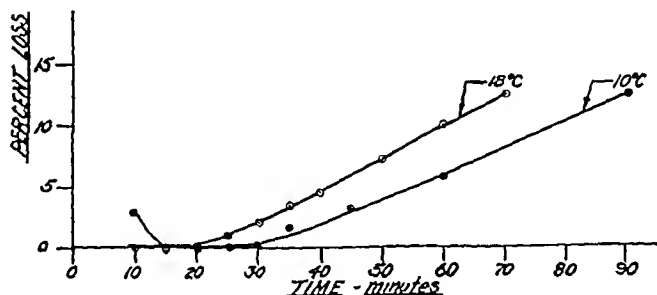


FIG 3 Effect of time on percentage color loss at constant temperature (10° and 18°)

ethylene glycol Our observations indicate that pyridine does not stabilize the color At constant sodium sulfide concentration the stability of the color seems to depend upon the temperature of the day On cool days the color is stable for at least 30 minutes whether pyridine is added or not It is not necessary therefore to add anything but to keep the temperature low This gives the additional advantage that the color may be developed in smaller volumes Fig 2 shows the effect of temperature on the stability of the color

Fig 3 shows the effect of time on the loss in intensity of color The

time is plotted against the percentage loss at constant temperature. It is apparent that the color should be read within 25 minutes after color development at 18° or between 15 and 60 minutes if developed at 10°. It will be noted that at low temperatures the color does not fully develop until about 15 minutes have elapsed. In view of these observations the sodium sulfide solution is kept in the refrigerator before use. The petroleum ether containing the pentabromoacetone is cooled to refrigerator temperature before the sodium sulfide is added. Subsequent operations are designed to keep the temperature below 15°.

By reading the colors at 450 m $\mu$  as compared to 650 m $\mu$  (where the absorption should be nil, see Fig. 1) it became apparent that an opalescence was present which caused errors as high as 100 per cent in dilute solutions. While this opalescence was apparent, once we knew of its existence, it could be easily overlooked. This opalescence can be eliminated by centrifuging at 2000 R P M. It is probably caused by emulsification of the petroleum ether with the sodium sulfide solution.

Several investigators have noted that the grade of petroleum ether is important. This difficulty is eliminated by purifying the petroleum ether with concentrated sulfuric acid and potassium permanganate, drying over anhydrous potassium carbonate, and then distilling.

The opalescence, lack of temperature control, and impurity of the petroleum ether could easily account for the variations described by Josephson and Hunter.

In order to shorten the time of the operation and in order to be able to develop the colors in smaller volumes, it was decided to eliminate the separatory funnels. The procedure is transferred, therefore, to glass-stoppered test-tubes. All washings are also eliminated by taking aliquots of the petroleum ether. In order to facilitate the taking of aliquots and to minimize losses due to evaporation, a higher boiling petroleum ether fraction (90–100°) is used. *n*-Heptane, b p 96–97°, may be purchased and used without purification.

In taking aliquots, it is necessary to centrifuge down the water adhering to the sides of the test-tubes. By this procedure a petroleum ether aliquot free of the aqueous layer is obtained. Since the solubility of water in high boiling petroleum ether is nil, the petroleum ether aliquot can be considered free of the lower layer and no washings are necessary. The aliquot is transferred to a second tube having a ground glass stopper. A desired amount of sodium sulfide is now added to develop the color. 3.5 ml are added if the color is to be read in the Coleman spectrophotometer or 1 ml or less if it is to be read in the Beckman spectrophotometer, depending upon the choice of the micro cuvettes employed.

It is of interest to note at this point that this technique can usually be



applied to any method in which separatory funnels are used and washings are necessary. By use of test-tubes centrifuging is simplified and a better separation of phases is obtained.

When the Coleman spectrophotometer is employed, special tubes with a 5 cm light path, containing 3 ml of liquid, are used. These were designed by A. E. Sobel for his vitamin A method.<sup>2</sup> These tubes are 5 cm horizontal absorption tubes adapted to the Coleman spectrophotometer. Their capacity is 3.0 ml. For extraction 15 cm Pyrex test-tubes with ground glass stoppers and marks at 2, 3, and 5 ml were used. These marks may be scratched on by the investigator since the volumes are not critical.

With these improvements the method is easily applicable to 1 ml of whole blood if the Coleman spectrophotometer is used or 0.3 ml of whole blood with the Beckman spectrophotometer. Correspondingly smaller quantities of serum and plasma may be used, for serum and plasma have higher concentrations of citric acid than whole blood. 2 to 60  $\gamma$  of citric acid are conveniently determined on the Beckman spectrophotometer and from 5 to 60  $\gamma$  on the Coleman spectrophotometer in the standard solutions.

### Methods

#### Reagents—

Citric acid. Stock solution, 1 ml = 1 mg of anhydrous citric acid (analytical reagent).

H<sub>2</sub>SO<sub>4</sub>, 18 N (analytical reagent).

Bromine water, saturated (analytical reagent).

Potassium bromide, 1 M (analytical reagent).

Hydrogen peroxide, 6 per cent, obtained by diluting 10 ml of 30 per cent H<sub>2</sub>O<sub>2</sub> (analytical reagent) with 500 ml of water. This solution should be kept in a refrigerator.

Sodium sulfide, 4 per cent solution, 40 gm. Sodium sulfide crystals, Na<sub>2</sub>S · 9H<sub>2</sub>O (analytical reagent), are diluted to 1000 ml. The solution should read 95 per cent transmission or better in a Coleman spectrophotometer against water at 450 m $\mu$ . When the sodium sulfide solution reads less than 95 per cent transmission, it should be discarded and a new solution prepared. This solution is kept in a refrigerator.

Petroleum ether, b.p. 90–100°. The commercial petroleum ether must be purified by the following procedure. About 700 ml of petroleum ether are shaken with about 100 ml of concentrated H<sub>2</sub>SO<sub>4</sub> in a 1 liter separatory funnel and allowed to stand overnight. The H<sub>2</sub>SO<sub>4</sub> is drawn off. The petroleum ether is then washed three times with 50 ml portions of H<sub>2</sub>SO<sub>4</sub>. It is now washed with 100 ml of water several times. The petroleum ether is then shaken with a saturated solution of KMnO<sub>4</sub>, made up in 0.5 H<sub>2</sub>SO<sub>4</sub>,

<sup>2</sup> Private communication.

biological fluid with this curve, the results obtained must be multiplied by 6/5 because originally a five-sixths aliquot was taken of the trichloroacetic acid filtrate. The curve as plotted from determinations on known amounts of citric acid actually represents only four-fifths of the total amount of citric acid present. This is so because a four-fifths aliquot of the petroleum ether, which contains the pentabromoacetone, is taken. Since the same procedure is followed on the trichloroacetic acid aliquot of the biological material, the results may be directly read from the curve for the aliquot.

### Results

The method was applied to whole blood, serum, plasma, and urine. For plasma the fresh blood is drawn into a paraffined tube and centrifuged while packed in ice. This was done to decide whether, for convenience, serum could be used routinely.

TABLE I  
*Determination of Citric Acid in Whole Blood, Serum, Plasma, and Urine*

Sample No	Whole blood	Whole blood recovery*	Serum	Plasma	Urine	Urine recovery*	Whole blood to plasma ratio
	mg per cent	per cent	mg per cent	mg per cent	mg per cent	per cent	
1	1.30	99	1.92	1.92	125.0	14	0.677
2	1.47	95	2.15	2.16	41.0	35	0.684
3	1.46	97	2.19	2.19	72.5	100	0.667
4	1.67	95	2.60	2.50	101.0	97	0.643
5	1.60	97	2.33	2.39	46.0	95	0.672

\* 0.2 mg of citric acid added (this is equivalent to increasing the blood citric acid by 2 mg per cent and the urine citric acid by 100 mg per cent)

Table I indicates some results on a series of determinations on fasting samples of whole blood, serum, plasma, and urine. Each value is the average of duplicates. The results obtained on duplicates varied from each other from 0 to 3 per cent.

It is apparent that for clinical determinations the method is adequate. The values for normal serum fall within a range described by Sjostrom (24). It is of interest to note that serum and plasma show slight variation within the experimental error. The clotting mechanism therefore does not remove appreciable amounts of citric acid. The ratio of the concentration of citric acid in whole blood to plasma is fairly constant at 64 to 68 per cent. This would indicate that substantially all the citric acid is in the serum. The hematocrit, as usually measured, gives a value of about 40 per cent. If there were no citric acid in the cells, the whole blood to plasma ratio

would be approximately 0.60. This is distinct from the observations on dogs, which have an appreciable amount of citric acid in the red cells (17). Nordbo and Schersten (25) report that human red blood cells contain practically no citric acid. For routine determinations serum determinations are therefore recommended.

No relationship is apparent between the citric acid concentration in urine and serum. This is in accordance with the observations of Östberg (26) and Lindquist (27). Concentrations in the urine are from 20 to 60 times the concentration in blood. The kidney apparently concentrates the citric acid. The excretion of citric acid in a 24 hour period is of the order of 1 to 1.5 gm.

The recoveries indicate that the method is accurate within 5 per cent for biological fluids.

#### SUMMARY

1. A method is described for the determination of citric acid in 1 ml. of whole blood, plasma, and serum, and 0.02 ml. of urine.

2. By this method amounts of the order of 2 to 60  $\gamma$  of citric acid can be determined with an accuracy of 5 per cent.

3. The method is rapid and requires only the usual apparatus found in a well equipped laboratory. At least twenty determinations can be completed in a working day.

4. The distribution of citric acid between whole blood, plasma, and serum was measured. This distribution indicates that practically all of the citric acid is in the plasma or serum. The clotting mechanism does not remove a measurable amount of citric acid from the serum.

5. No relationship was observed between the citric acid concentration in blood and urine.

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gm had to be excluded because the diaphragms were too thick to allow complete oxygen diffusion

*Production of Diabetes*—The rats were fasted 24 hours, then injected intravenously with 50 to 60 mg per kilo of alloxan monohydrate (Eastman Kodak), the drug was employed as a fresh 5 per cent solution in water ether anesthesia to immobilize the rats was found to expedite the injections

After injection, the animals were returned to the stock diet, with water to drink Blood sugars were obtained on the fed animals at 48 hours and,

TABLE I

*Blood Sugar Values Obtained after Intravenous Administration of 50 to 60 Mg per Kilo of Alloxan Monohydrate to Rats of Inheuser-Busch (1B) and Sprague Dawley (SD) Strains*

Strain	Range of blood sugar values	Per cent of rats found in each blood sugar group*	
		Fed	Fasting
AB	<i>mg per cent</i>		
	0-99	2 (1)	8 (3)
	100-299	0	30 (12)
	300-499	51 (26)	53 (21)
	500-699	36 (17)	5 (2)
	700-899	6 (3)	2 (1)
SD	900 up	2 (2)	2 (1)
	0-99	3 (1)	27 (8)
	100-299	8 (3)	53 (16)
	300-499	31 (12)	13 (4)
	500-699	38 (15)	7 (2)
	700-899	15 (6)	0
	900 up	5 (2)	0

\* The figures in parentheses give the actual number of rats The total number of fasting animals is less than the total of fed animals because of loss during fasting

after a 16 to 20 hour fast, at 72 hours after injection Blood sugars on fed and fasted animals were determined occasionally thereafter until the animals were killed, the blood sugar values given in the tables for fasted animals are those obtained on the day the animal was killed for use of the diaphragm

The blood sugar values obtained upon all surviving rats given 50 to 60 mg per kilo of alloxan monohydrate are summarized in Table I

There are apparently differences in the susceptibility of different strains of rats to alloxan, as shown by the blood sugar levels during fasting The differences are less marked when the blood sugars of fed animals are compared This would indicate that blood sugar determinations on fed animals are not an adequate criterion of the severity of alloxan diabetes in

rats. This point is perhaps worth stressing, as blood sugars of allovan-treated rats are almost invariably reported in the literature for the fed animals.

Blood sugars were determined in duplicate on 0.05 ml. samples obtained from the tails of unanesthetized animals. The blood was allowed to drop on a chilled watch-glass, from which it could be readily pipetted without clotting. Analyses were performed by the Nelson method (4) with a 540 m $\mu$  filter and the Klett-Summerson photoelectric colorimeter.

*Removal of Adrenals*—Each rat received 10 mg. of atropine sulfate in 0.5 ml. of 0.9 per cent sodium chloride solution subcutaneously, this dose, given 30 to 120 minutes prior to operation, was found useful in reducing respiratory complications produced by ether.

Bilateral adrenalectomy was performed under ether anesthesia according to the technique described by Ingle and Griffith (5). The animals were then given the stock diet, with 1 per cent sodium chloride solution to drink, until killed.

Adrenalectomy of the diabetic rats was followed by a fall of the blood sugar of the fed animals from 300 to 800 to about 200 to 400 mg. per cent, these diabetic-adrenalectomized rats could not be safely fasted for 16 to 20 hours, as they tended to die of hypoglycemia. On this account, a fasting period of 6 hours was employed, in this period the blood sugar fell to normal values or below, in contrast to the blood sugar of more than 300 mg. per cent after a fast of 16 to 20 hours in the same diabetic animals prior to adrenalectomy.

*Suspension Medium for Diaphragms*—Krebs-Henseleit solution (6) was used in all experiments. This was chosen because of its similarity to extracellular fluid with respect to ionic content. In all experiments, except those specifically noted, the gas phase was 95 per cent oxygen-5 per cent carbon dioxide, giving an initial pH of 7.4 to the medium.

Gemmell and Hamman (7) found that the glucose utilization by rat diaphragm was higher with 300 or 500 mg. per cent of glucose in the medium than with 200 mg. per cent, they also found that the absolute increase in glucose utilization produced by insulin was somewhat larger at 300 or 500 mg. per cent than at 200 mg. per cent.

For the present investigation, two concentrations of glucose were tried, 100 and 200 mg. per cent. Although the rate of glucose utilization and the effect of insulin tended to be somewhat higher at 200 than at 100 mg. per cent, the lower concentration was chosen for most of the experiments in order to compare rates of glucose utilization at a substrate concentration corresponding to the normal blood sugar level. To allow the stimulation by insulin to be demonstrated clearly, the weight of the diaphragm sample was limited to about 30 mg. so that the sugar concentration in the medium

would not fall below 50 mg per cent at the end of the incubation period. In addition, a few experiments were run at 200 mg per cent.

The solutions containing insulin were prepared by diluting 0.1 ml of commercial (80 units per ml)letin (insulin, Lilly) with 8 ml of ice-cold medium. Control experiments showed that the 0.2 per cent phenol contained in the original insulin solution was sufficiently diluted in the final analyses so as not to give a color with the phosphomolybdate used in the glucose determinations. The final concentration of insulin to which the tissue was exposed was 0.05 mg per ml, which is approximately 1 unit per ml.

*Procedure for Handling Diaphragms*—After the animals were stunned, the diaphragm was removed and immersed immediately in ice-cold Krebs-Henseleit solution previously equilibrated with 95 per cent oxygen-5 per cent carbon dioxide and containing 100 mg per cent of glucose, during this operation, stretching and handling of the diaphragm were kept at a minimum and care was taken to avoid the bleeding which results from puncturing the posterior vena cava or the liver.

The muscle of the diaphragm was trimmed to remove the central tendon and the extremely thin muscle at the periphery. Each half of the diaphragm was then cut lengthwise to yield two pieces and again subdivided when necessary.

Each piece was blotted separately, weighed on a torsion balance, and transferred to the chilled medium contained in the side arm of a Warburg vessel or in a small round bottom cylindrical vessel prepared for the purpose by sealing off one end of a 5 cm length of 16 mm Pyrex tubing. The medium (0.35 to 0.36 ml) was previously placed in the vessels by means of a Lang-Levy micro pipette, micro pipettes delivering 0.09 to 0.115 ml were used for the sampling at the end of the experiment. The vessels containing medium and tissue were next attached to Warburg manometers, equilibrated with the gas phase, placed in a bath at 37°, and shaken for 2 hours at 96 cycles per minute. At the end of the 2 hour period, the vessels were removed, stoppered, and chilled in ice water.

For analysis of residual glucose in the medium, duplicate 0.09 to 0.115 ml aliquots were pipetted into 1.5 ml of water and precipitated with 0.2 ml each of barium hydroxide and zinc sulfate, 1.0 ml samples of the filtrate were analyzed by the Nelson method. Samples of the original medium were carried through the analysis concurrently, and the glucose utilization was determined by difference. In order to appraise the significance of the differences in glucose utilization between diaphragms from various types of animals,<sup>2</sup> the results were analyzed statistically by standard procedures. The values of *P* were taken from the Fisher *t* table (8), according to this

<sup>2</sup> The experiments on different groups of animals were carried out on overlapping dates in order to minimize accidental variations in preexperimental conditions.

method of expression, a value of  $P = 0.01$  means that the difference observed would occur by chance only once in a hundred trials, etc

Since the blood sugar of the diabetic rats was so much higher than that of the normal rats, there was the possibility that extra glucose in the diabetic diaphragms might lead to an underestimation of the rate of glucose utilization. Accordingly, the free sugar content was determined in the diaphragms of both normal and diabetic animals. After being chilled as usual in ice-cold medium containing 100 mg per cent of glucose, the tissue was blotted, weighed, and thoroughly ground with sand in a known volume of water, this was followed by precipitation of protein with barium hydroxide and zinc sulfate. The clear filtrate was then analyzed by the Nelson method. Four normal diaphragms contained an average of 0.40  $\gamma$  of glucose per mg of wet tissue, while five diabetic diaphragms (from animals with blood sugars ranging from 380 to 920 mg per cent) contained an average of 0.39  $\gamma$  of glucose per mg of wet tissue. Thus, owing to the preliminary washing, no extra sugar was carried over by the diabetic diaphragms into the suspension medium.

### Results

All rates of glucose utilization are expressed as mg per gm of wet diaphragm per hour. An initial glucose concentration of 100 mg per cent in the suspension medium was employed for all experiments reported in the tables. Tests of the significance of differences between the rates for the various groups of diaphragms are summarized with the data in the appropriate tables.

*Diaphragms from Normal Rats*—The mean rate of glucose utilization was found to be 1.93 mg per gm without insulin and 2.47 with insulin (Table II). When expressed in the same units as are used here, the values obtained by Stadie (9) at 100 mg per cent initial glucose were 1.91 without insulin and 3.48 with insulin. The corresponding values obtained by Gemmill and Hamman (7) at 200 mg per cent initial glucose were 1.80 and 3.44. The reason for the smaller insulin stimulation here observed is not known, but may lie in the difference in ionic content of the suspension media, Stadie used a "saline-phosphate" solution, Gemmill used a magnesium-free solution buffered with phosphate, in the present experiments, the solution contained magnesium in approximately the concentration found in extracellular fluid, and was buffered with carbon dioxide-bicarbonate.

Since the same diaphragm was cut into a number of pieces, some of which served as controls, while others were used for the measurement of stimulation by insulin, it was of importance to find out how much variation in glucose uptake was observed among individual pieces. Data bearing on this point are included in Tables II and III, they show that the method

of cutting the diaphragm into a number of pieces introduces no serious error and is useful for testing the effect of addition of various substances

One diaphragm from a normal rat, in which 95 per cent  $N_2$ -5 per cent  $CO_2$  instead of 95 per cent  $O_2$ -5 per cent  $CO_2$  was used as the gas phase, showed a very low rate of glucose utilization, approximately 10 per cent of the rate in oxygen

TABLE II

*Glucose Uptake by Diaphragms of Normal Rats*

Each line gives measurements on diaphragm from one rat. The number in parentheses gives the number of pieces from each diaphragm used in parallel experiments. Each piece was run separately and the values obtained were averaged. The standard deviation is included when more than two pieces were used.

Rat No	Strain	Fasted (Fa) or fed (F) prior to use	Glucose uptake, mg per gm wet tissue per hr	
			In glucose	In glucose + insulin
1	AB	F	2.13 (8) $\pm$ 0.06*	3.52 (2)
2	"	Fa	2.46 (7) $\pm$ 0.04*	
3	"	F	2.34 (2)	
4	"	"	1.52 (6) $\pm$ 0.08*	
5	SD	"	2.18 (5) $\pm$ 0.12*	2.57 (2)
6	"	Fa	2.18 (2)	
7	"	F	1.71 (2)	
8	AB	Fa	1.58 (2)	
9	"	"	1.43 (2)	1.72 (2)
10	"	"	1.91 (2)	2.38 (2)
11	"	"	1.98 (2)	3.14 (1)
12	"	"	2.01 (2)	2.64 (1)
13	"	"	1.61 (2)	2.09 (2)
Mean			1.93	2.47

Test for significance of insulin stimulation (pair comparison),  $t = 4.6$ ,  $P = 0.002$

\* Standard deviation

*Diaphragms from Diabetic Rats*—The rate of glucose utilization by diaphragms from diabetic, Strain AB, rats was found to depend upon the severity of the diabetes. Diaphragms from rats which had blood sugars below 200 mg per cent in the fasted state were normal with respect to the rate of glucose utilization, while every diaphragm from a rat with a fasting blood sugar above 300 mg per cent exhibited a depressed rate (Table III). The mean value for twelve animals of this latter group was 0.80, a decrease of 59 per cent in the rate of sugar utilization. Addition of insulin increased the rate of utilization by diabetic diaphragm to 1.38 mg per gm. This represented an absolute stimulation of 0.58 mg per gm per hour, as com-



pared to an absolute stimulation of 0.54 mg per gm per hour with normal diaphragm

The fact that the diabetic diaphragm treated with insulin used less glucose than the normal diaphragm raised the question of the total hexokinase

TABLE III

*Glucose Uptake by Diaphragms of Diabetic Rats, Strain AB*

The conventions and abbreviations are the same as in Table II. The animals were fasted for 16 to 20 hours prior to the experiment

Rat No	Days after alloxan injection	Blood sugar		Glucose uptake mg per gm wet tissue per hr	
		Fed	Fasting	In glucose	In glucose + insulin
Group A Rats with fasting blood sugars of 300 mg % or more and depressed glucose uptake					
		mg per cent	mg per cent		
1	3	592		0.85 (6) $\pm$ 0.04*	
2	3	441	400	0.88 (8) $\pm$ 0.04*	
3	3	1000		0.45 (6) $\pm$ 0.04*	
4	3	584		0.92 (6) $\pm$ 0.08*	
5	3	468	336	0.76 (2)	1.16 (2)
6	3	512	336	0.73 (2)	1.26 (2)
7	3	540	412	0.88 (2)	1.35 (2)
8	5	696	412	1.00 (2)	1.40 (2)
9	5	456	360	0.46 (2)	1.33 (2)
10	3	680	720	0.60 (2)	1.18 (2)
11	4	540	344	1.06 (2)	1.37 (2)
12	4	580	428	1.00 (2)	1.95 (2)
Mean				0.80	1.38

Test for significance of insulin stimulation (pair comparison)  $t_{6,8}, P < 0.001$

" " " " difference between mean for normal diaphragm (without insulin) and mean for diabetic diaphragms of Group A (without insulin)  $t_{10,3}, P < 0.001$

Group B Rats with fasting blood sugars below 300 mg %					
1	15	376	152	1.49 (2)	1.99 (2)
2	11	440	92	1.64 (2)	2.14 (2)

\* Standard deviation

content of the muscle from alloxan-diabetic rats. Leg muscles of five rats (Nos 7, 8, 9, 11, 12, Table III) were ground and thoroughly extracted with water, the extract was allowed to stand for about 1 hour at 0° (during which an inhibitory factor present in fresh extracts is destroyed (3)) and the hexokinase activity tested as described in a previous paper (3). In no instance was the hexokinase activity below the normal range.

The rate of glucose utilization by diaphragms from diabetic rats of Strain SD was also dependent upon the severity of the diabetes but, as stated previously, it was difficult to make rats of this strain as severely diabetic as those of Strain AB. Diaphragms from three out of five animals with high blood sugars have shown depressed rates, while diaphragms from five animals with fasting blood sugars between 200 and 300 mg per cent had rates which were within the normal range (Table IV).

TABLE IV

*Glucose Uptake by Diaphragms of Diabetic Rats, Strain SD*

The conventions and abbreviations are the same as in Tables II and III

Rat No	Time after alloxan injection	Blood sugar		Glucose uptake mg per gm wet tissue per hr	
		Fed	Fasting	In glucose	In glucose + insulin
Group A Rats with fasting blood sugars of 300 mg % or more, or depressed glucose uptake					
	days	mg per cent	mg per cent		
1	2	960		0.63 (5)	
2	2	516		0.30 (2)	
3*	3	512	512	0.68 (1)	1.58 (1)
4	3	880	488	2.61 (2)	3.07 (1)
5	16	460	460	1.90 (2)	2.65 (2)
Group B Rats with fasting blood sugars below 300 mg % *					
1	5	580	256	1.49 (2)	2.13 (1)
2	1	440	248	1.42 (2)	1.97 (2)
3	3	628	240	2.14 (2)	3.12 (1)
4	5	528	212	1.76 (2)	2.27 (2)
5	4	408	212	1.59 (2)	2.18 (2)
Mean, Group B				1.68	2.33

Test for significance of insulin stimulation (pair comparison)  $t = 9$ ,  $P = 0.003$

\* This rat was fed high protein diet (11) for 6 days, then fasted 24 hours prior to injection of 50 mg of alloxan monohydrate per kilo.

*Diaphragms from Severely Diabetic Rats Which Were Subsequently Adrenalectomized*—It has been reported (3) that the inhibition of the hexokinase reaction in muscle extracts of diabetic rats is increased by the addition of adrenal cortical extract. Conversely, an amelioration of diabetic symptoms in various species has been observed after adrenalectomy (see Long *et al.* (10) for review). It was of interest to see whether this observation on the whole animal could be duplicated on isolated muscle.

Since every diaphragm taken from diabetic, Strain AB, rats with blood sugars above 300 mg per cent in the fasting state had shown a significantly depressed rate, animals were taken from the same group for adrenalectomy (Table V)

Adrenalectomy was followed by a fall in blood sugar in six of seven fed animals. During even short periods of fasting, there was a marked drop in blood sugar in every animal. In Rat 2, for example, the blood sugar

TABLE V

*Glucose Uptake by Diaphragms of Diabetic-Adrenalectomized Rats, Strain AB*

Each rat of this group showed a fasting blood sugar over 300 mg per cent before adrenalectomy. The conventions and abbreviations are the same as in Tables II and III.

Rat No	Blood sugar				Time of use		Glucose uptake mg per gm wet tissue per hr	
	Before adrenalectomy		After adrenalectomy		After alloxan	After adrenalectomy	In glucose	In glucose + insulin
	Fed	Fasting	Fed	Fasting				
	mg per cent	mg per cent	mg per cent	mg per cent	days	days		
1	356	336	348	12*	12	3	2.41 (2)	3.37 (2)
2	416	380	296	40†	13	9	1.98 (2)	2.38 (2)
3	72S	440	280	108†	13	9	2.08 (2)	1.80 (2)
4	66S	440	240	96†	8	3	2.20 (1)	3.12 (1)
5	32S	336	392	98†	10	4	1.76 (2)	2.86 (2)
6	60S	380	348	180†	11	3	2.74 (2)	3.86 (2)
7	552	424	404	20†	11	3	2.08 (2)	2.78 (2)
Mean							2.18	2.88

Test for significance of insulin stimulation (pair comparison)  $t_{34}$ ,  $P < 0.01$

\* Fasted 24 hours, occasional convulsions occurred after this period of fasting.

† Fasted 6 hours, this period was chosen to minimize the loss of animals which was incurred with longer fasting.

fell from 296 to 40 mg per cent after only 6 hours of fasting, before adrenalectomy, the same animal had a blood sugar of 380 mg per cent after 20 hours of fasting.

The rate of glucose utilization in this group was 2.18 mg per gm, as compared to the values of 0.80 and 1.93 mg per gm for the diabetic and normal groups, respectively. Thus, a depressed glucose utilization in diaphragms from diabetic animals does not persist after adrenalectomy.

*Diaphragms from Adrenalectomized Rats*—The rates of glucose utilization for diaphragms from eight adrenalectomized rats were 2.03 mg per gm without insulin and 2.40 with insulin (Table VI). The rate without insulin

is very close to that for normal diaphragm. No previous measurements of glucose utilization by diaphragm from adrenalectomized animals are available, but Koepf, Horn, Gemmill, and Thorn (12) stated in an abstract that "in a medium of 0.2 per cent glucose, the increase in glycogen content of the diaphragm of both normal and adrenalectomized rats was of the same magnitude."

TABLE VI

*Glucose Uptake by Diaphragms of Adrenalectomized Rats*

The conventions and abbreviations are the same as in Tables II and III

Rat No	Strain	Fasting blood sugar after adrenalectomy	Time of use after adrenalectomy	Glucose uptake, mg per gm wet tissue per hr	
				In glucose	In glucose + insulin
		mg per cent	days		
1	SD	58	3	1.81 (2)	2.01 (2)
2	"	60	5	1.47 (2)	1.83 (1)
3	"	58	25	2.37 (2)	2.89 (2)
4	"	60	23	1.85 (1)	1.80 (1)
5	AB	56	4	1.61 (2)	2.00 (2)
6	"	44	7	2.12 (2)	2.29 (2)
7	"	44	7	2.54 (2)	3.45 (2)
8	"	28	8	2.45 (2)	2.90 (2)
Mean				2.03	2.40

Test for significance of insulin stimulation (pair comparison)  $t = 3.7$ ,  $P = 0.003$

It is worthy of note that the insulin stimulation observed with the two adrenalectomized groups is smaller than that for other groups.

## DISCUSSION

The results reported in this paper show that the isolated diaphragm can serve as an indicator of the changes in carbohydrate metabolism observed in the intact animal. Severe diabetic hyperglycemia is associated with a decreased glucose utilization by the isolated muscle, and amelioration of diabetes by adrenalectomy is paralleled by a return of the glucose utilization of the diaphragm to normal.

For reasons stated in the introduction, the rate of glucose uptake by the isolated diaphragm is a measure of the activity of the hexokinase system. It would appear that this system is not fully active in diaphragms removed from either fed or fasted normal rats, since addition of insulin increases the glucose uptake.

In diaphragms of diabetic rats, insulin did not restore glucose utilization to normal. Since the total hexokinase content of diabetic muscle, as measured in muscle extracts, was found to be within the normal range, the failure of insulin to restore normal utilization in the intact diabetic muscle may be due to unfavorable conditions for the action or penetration of the hormone. The following observation may be significant in this respect. Nelson and Corkill and Nelson (13) have shown that insulin fails to exert a stimulatory effect on the glucose uptake and on glycogen formation in the diaphragm of normal rats previously injected with anterior pituitary extract.

Further work with the isolated diaphragm may throw additional light on the postulated relationships of anterior pituitary, adrenal cortex, and insulin to the hexokinase system.

#### SUMMARY

1 Comparisons have been made between rates of glucose utilization by isolated diaphragms from the following groups of rats: (a) normal, (b) diabetic, (c) diabetic, subsequently adrenalectomized, and (d) adrenalectomized. The effect of insulin upon glucose utilization by diaphragms of each group has also been studied.

2 The rate of glucose utilization by diaphragms from diabetic rats is dependent upon the degree of the diabetes; it decreases below normal as the severity of the diabetes, as measured by the fasting blood sugar, increases. With the strain of rats most extensively studied, every diaphragm from rats having fasting blood sugars above 300 mg per cent exhibited a rate of glucose utilization significantly lower than normal, the average for diaphragms from twelve such rats being 59 per cent below the normal rate. Adrenalectomy of such severely diabetic rats is followed by a fall in the blood sugar level of the fed animals, an inability to maintain high blood sugar levels even during short periods of fasting, and return of the rate of glucose utilization by the diaphragm to normal.

3 The glucose utilization by diaphragms from adrenalectomized rats is within the normal range.

4 The severity of diabetes, as judged by the blood sugar level during fasting, differed markedly in two strains of rats.

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# OLEIC ACID AND RELATED COMPOUNDS AS GROWTH FACTORS FOR LACTIC ACID BACTERIA\*

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(Received for publication, June 20, 1947)

It is well known that under special conditions certain fatty acids markedly affect growth of lactic acid bacteria. In the microbiological assay of riboflavin and of pantothenic acid, marked stimulation of the assay organism, *Lactobacillus casei*, results from the presence of small amounts of lecithin or of fatty acids (1, 2) when these vitamins are present in sub-optimal amounts. At higher levels of the fatty substances, inhibition may result. The same effect of lipoidal materials is observed in the assay of pantothenic acid with *Lactobacillus arabinosus* (3). More recently it was observed that the presence of oleic acid (4), rice oil (4), or a fat-soluble substance of unknown nature obtained from blood (5) eliminates the requirement of *Lactobacillus casei* for biotin. Oleic acid also duplicates, in many respects, the growth-promoting action of acetate for a variety of lactic acid bacteria (6).

In surveying the nutritive requirements of various lactic acid bacteria, a number of cultures were encountered for which oleic acid or related compounds are essential growth factors, even in the presence of both biotin and acetate. The present study deals with these organisms and extends the studies on lactic acid bacteria for which fatty acids can substitute for biotin.

## EXPERIMENTAL

### *Organisms for Which Fatty Acids Are Essential*

**Stock Cultures and Inocula**—The cultures used were obtained from Professor W. B. Sarles, Department of Agricultural Bacteriology, University of Wisconsin, and were tentatively designated as *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, and *Streptococcus lactis*. The cultures had been carried on litmus milk since isolation and this practice was continued. Biweekly transfers were made, following

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from Merck and Company, Inc., and from the Schenley Research Institute.

transfer, cultures were incubated at 37° until coagulation and acid formation indicated good growth (24 to 48 hours), and were then held in the refrigerator for the remainder of the 2 week period

Inoculum medium was prepared by supplementing each 5 cc of the basal medium (described below) with 100 mg of lactose, 1 mg of cysteine, 10 mg of Tween 80 (a polyoxyethylene derivative of sorbitan monooleate), 4 cc of whey (lactalbumin removed by heating to 100°), and sufficient water to make 10 cc. This was put into tubes in 10 cc lots and sterilized by autoclaving. To prepare inoculum, a loop of the refrigerated litmus milk culture was transferred to 10 cc of this inoculum medium, incubated 24 hours at 37°, the cells separated by centrifuging, and the supernatant medium replaced by an equal volume of sterile 0.9 per cent sodium chloride solution. 1 drop of this heavy suspension of cells was used to inoculate each tube.

*Basal Medium*—The basal medium was that described by Roberts and Snell (7) with the content of inorganic salts arbitrarily lowered (0.1 cc of Salts B<sup>1</sup> per 10 cc of final medium was substituted for Salts C of the medium described). The double strength medium was prepared in lots of 1 liter each, stored at 4°, and used over a period not longer than 1 month. Immediately before use, each 5 cc of this double strength medium was supplemented with 10 mg of Difco yeast extract, 1 mg of cysteine, and 100 mg of lactose.

*Procedure*—The procedure is that commonly used in microbiological assay for vitamins or amino acids. The supplemented basal medium is dispensed in 5 cc lots to a series of selected, rimless 18 × 150 mm Pyrex test-tubes. Aliquots of the test solutions are added and the volume in each tube adjusted to 10 cc with distilled water. The tubes are plugged with cotton, autoclaved at 15 pounds pressure for 6 minutes, cooled, inoculated, and incubated at 37° for an appropriate period. Growth is estimated turbidimetrically directly in the culture tubes with an Evelyn photoelectric colorimeter equipped with an adapter for this size tube. Growth data for each experimental series were usually recorded at 24, 48, and 72 hours. In some cases the organisms failed to develop maximally in 24 hours, but growth was usually maximum at 48 hours. Readings at 72 hours showed essentially no change.

*Results*—Preliminary experiments showed that little or no growth of these organisms occurred in the basal medium unless lipoidal materials, such as commercial lecithin, were added. Oleic acid is known to be an essential growth factor for a few bacteria belonging to other genera (8), and was therefore suspected as the active material. At relatively low

<sup>1</sup> Salts B contains 10 gm of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.5 gm of NaCl, 0.5 gm of FeSO<sub>4</sub> · 7H<sub>2</sub>O, 0.5 gm of MnSO<sub>4</sub> · 4H<sub>2</sub>O, and water to make 250 cc.



levels, however, unsaturated fatty acids are known to be toxic to many lactic acid bacteria. The use of the Tweens (fatty acid esters of polyoxyethylene derivatives of sorbitan, sold by the Atlas Powder Company (9)), found by Dubos (10, 11) to supply fatty acids for some organisms in a

TABLE I

*Essential Nature of Oleic Acid or Related Substances for Growth of Certain Lactic Acid Bacteria\**

Additions to basal medium	<i>Lactobacillus acidophilus</i>	<i>Lactobacillus bulgaricus</i>	<i>Lactobacillus helveticus</i>	<i>Streptococcus lactis</i> †
None	—	—	—	—
Oleic acid (100 $\gamma$ )	+	+	+	+
Lecithin (500 $\gamma$ )	+++	+++	+++	+++
Tween 20‡	+++ (40 mg)	+++ (3 mg)	+++ (40 mg)	+
" 40‡ (10 mg)	—	—	—	—
Tween 60‡ (10 mg)	—	—	—	—
Tween 80‡	+++ (10 mg)	+++ (0.3 mg)	+++ (1.0 mg)	+++ (40 mg)
" 85‡	+++ (0.1 mg)	+++ (0.1 mg)	+++ (0.1 mg)	+
Saponin (10 mg)	+++	+	+	+
Tween 40 (10 mg) + oleic acid (100 $\gamma$ )	+++	+++	+++	+++
Saponin (10 mg) + oleic acid (100 $\gamma$ )	+++	+++	+++	+++

\* — indicates no growth, + slight growth, and +++ heavy growth after 72 hours incubation. The amount of test substance added to 10 cc of medium to secure the indicated growth response is given in parentheses.

† Yeast extract was omitted from the medium with this organism.

‡ The Tweens are polyoxyethylene derivatives of sorbitan esterified with fatty acids. The esterified fatty acids contained in each product (9) are the following: Tween 20, lauric acid; Tween 40, palmitic acid; Tween 60, stearic acid; Tweens 80 and 85, oleic acid.

convenient, water-soluble form, suggested itself. The results of exploratory experiments on the utilization of oleic acid, lecithin, and various Tweens by the test organisms are shown in Table I. In the absence of a source of unsaturated fatty acids, none of the organisms grew. Oleic acid itself supported only slight growth at the level tested. Lecithin permitted heavy growth. Heavy growth was also supported by the oleic acid-

containing Tweens (Tween 80 and 85), and also by lauric acid-containing Tween 20. Results with *Lactobacillus bulgaricus*, cited below, show that lauric acid is inactive for this organism, so that the activity of Tween 20 is perhaps due to contamination with utilizable fatty acids. Tweens 40 and 60, containing palmitic and stearic acid, respectively, were inactive. That the growth-promoting substance is actually oleic acid is shown by the fact that this substance is highly active for all organisms when tested together with Tween 40, which by itself, is inactive. We attribute the low activity of oleic acid, when tested alone, to its toxicity. Its toxic properties are eliminated by the inactive Tween, and its true growth-promoting properties then become apparent. Saponin (Eastman, "practical") shows slight activity for some of the organisms and eliminates the toxic action of oleic acid for others. A similar detoxifying action of saponins on oleic acid was noted by Hutner (12) with *Erysipelothrix rhusio pathiae* for which oleic acid is likewise an essential growth factor. With the latter organism, saponin alone was ineffective in promoting growth, its slight activity for the organisms studied here may be due to contamination with unsaturated fatty acids.

For more detailed study, *Lactobacillus bulgaricus* was selected. Williams and Fieger (4) have shown that the pH of the basal medium affects the efficiency with which oleic acid replaces biotin for *Lactobacillus casei*. Fig 1 shows the effect of the initial pH of the medium on the response of *Lactobacillus bulgaricus* to oleic acid. The pH was measured in each case after the medium had been autoclaved. At pH 5.0, 5.6, and 7.0 the growth response to oleic acid was negligible, only the response obtained at pH 7.0 is shown in Fig 1. At pH 6.0 to 6.5, oleic acid promotes growth at low levels, but at higher levels its toxic action completely obscures its growth-promoting effects. This toxic action becomes evident considerably before maximum growth of the organism is achieved. It is interesting to note that it would easily be possible completely to overlook the growth promoting properties of oleic acid, an essential substance, simply by testing at a level slightly removed from optimal.

The marked effect of Tween 40 (10 mg per 10 cc), itself inactive, in promoting utilization of oleic acid is shown in Fig 2. Optimal utilization occurred in this case when the initial pH was 7.0, considerable utilization occurred also at pH 5.6, but not at 5.0. The presence of Tween 40, therefore, extends greatly the pH range over which oleic acid is utilized, within this range it renders excess oleic acid wholly non-toxic.

In Fig 3, the growth response to Tween 80 at various initial hydrogen ion concentrations is shown. Again utilization occurs between pH 5.6 and 7.0, but not at 5.0. The activity of Tween 80 appears due chiefly to esterified oleic acid, since this product is 8 to 10 per cent as active for

*Lactobacillus bulgaricus* as is oleic acid when the latter is tested in the presence of inactive Tween 40 (cf Figs 2 and 3) Davis and Dubos<sup>1</sup>(11)

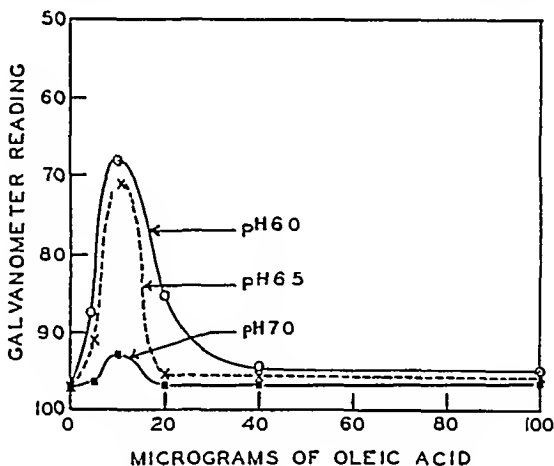


FIG 1 The effect of initial pH of the medium on the response of *Lactobacillus bulgaricus* to oleic acid

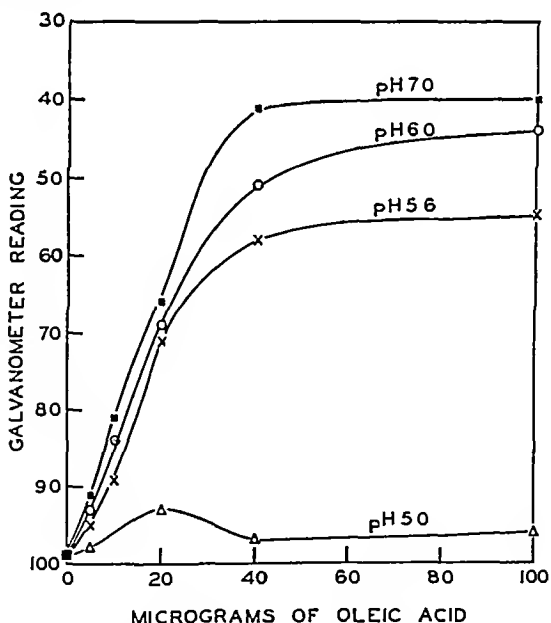


FIG 2 The effect of Tween 40 and initial pH of the medium on the utilization of oleic acid by *Lactobacillus bulgaricus*

found an average of only 0.6 per cent of free oleic acid in several different lots of Tween 80

The growth-promoting properties for *Lactobacillus bulgaricus* of a series of fatty acids were investigated. Capric, caprylic, lauric, myristic, and palmitic acids were inactive at levels from 0.1 to 1.0 mg per 10 cc, both in the presence and absence of Tween 40. Linoleic acid was only slightly less active than oleic acid.<sup>2</sup> The inactivity of lauric acid under these conditions indicates that the growth-promoting action of Tween 20 (Table I) is due to impurities. Products effective in replacing oleic acid had the following approximate relative activities (oleic acid = 100) when tested

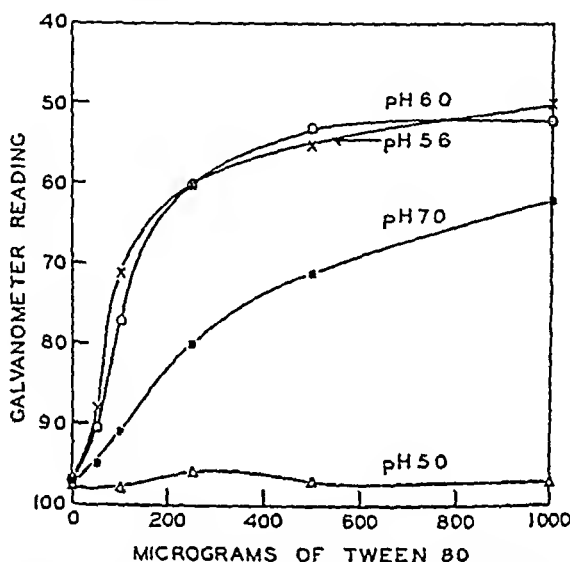


FIG. 3. The effect of initial pH of the medium on the utilization of Tween 80 by *Lactobacillus bulgaricus*.

under comparable conditions: linoleic acid, 80; lecithin, 10; Tween 80, 10; saponin, 0.1. The activity of both lecithin and Tween 80 is explicable on the basis of their contents of unsaturated fatty acids. It is possible that the very low activity of saponin is also due to contaminating traces of these fatty acids.

#### Organisms for Which Oleic Acid Is Essential Only in Absence of Brown

*Stock Cultures, Basal Media, and Inocula*—*Lactobacillus arabinosus* 17-5, *Lactobacillus casei*, *Lactobacillus delbrueckii* 3, *Leuconostoc mesenteroides* P-60, and *Streptococcus faecalis* R were carried by monthly transfer in yeast dextrose agar. *S. faecalis* was incubated at 30°, other organisms

<sup>2</sup> We are indebted to Professor H. A. Schuette for samples of these pure fatty acids.

at 37° The basal medium and technique used with *S faecalis* were those described by Rabinowitz and Snell (13) The basal medium used with

TABLE II  
Biotin Fatty Acid Relationships for Lactic Acid Bacteria

Addition to biotin deficient medium		<i>S faecalis</i> <i>L arabinosus</i> <i>L delbrueckii</i> <i>L cases</i> <i>L mesenteroides</i>									
		Galvanometer readings*									
			Tween 40, 10 mg per 10 cc		Tween 40, 10 mg per 10 cc		Tween 40, 10 mg per 10 cc		Tween 40, 10 mg per 10 cc		Tween 40, 10 mg per 10 cc
Biotin	mg per 10 cc										
	0 0	92	92	79		97		84		78	
	0 1	85	88	68		90		68		63	
	0 3	74	72	55		82		60		43	
	1 0	55	53	40		61		48		40	
Tween 80	10 0	39	39	31		28		36		42	
	mg per 10 cc										
	0 0	92		79		97		84		78	
	0 1	91		80		94		78		74	
	0 3	67		67		78		62		64	
Tween 40	1 0	50		49		54		44		50	
	10 0	40		39		31		35		43	
	0 0	92		79		97		84		78	
	0 3	92		81		100		85		80	
	1 0	97		76		100		81		79	
Oleic acid, initial pH 7 0	10 0	91		61		97		71		68	
	γ per 10 cc										
	0	92	92	79	61	97	97	84	71	78	74
	10	89	87	89	55	90	76	100	51	77	49
	30	73	59	78	47	80	45	100	34	65	47
Oleic acid, initial pH 5 5	100	63	48	62	39	57	29	99	25	41	49
	300	54	50	48	41	41	28	98	24	40	47
	0	92	92	79	61	97	97	84	71	78	74
	10	89	76	92	52	96	56	91	47	88	54
	30	82	69	80	41	82	43	81	36	77	54
	100	68	69	57	32	52	27	82	27	54	57
	300	67	66	37	33	38	25	87	27	62	57

\* 24 hour readings for *Streptococcus faecalis*, all other readings after 48 hours incubation The uninoculated medium was set at 100 per cent light transmission

the other organisms was essentially that of MacLeod and Snell (14), but with acid-hydrolyzed casein substituted for the enzymatic casein digest of the former medium In both media, biotin was omitted, and the casein hydrolysate was treated with 10 per cent of activated charcoal (Darco

G-60) for 15 minutes at pH 3.0, filtered, and then treated a second time at pH 7.0. Cystine and pyridoxamine were added aseptically following the separate autoclaving of the medium (13).

Inocula were grown for 24 to 48 hours in 10 cc of the appropriate basal medium supplemented with biotin (0.01  $\gamma$  per 10 cc). The cultures were centrifuged, the supernatant medium replaced with 10 cc of sterile saline, and the organisms resuspended. 4 drops of this suspension were

TABLE III

*Response of Streptococcus faecalis to Biotin or Oleic acid in Presence of biotin*

Oleic acid	Egg white preparation*	Galvanometer readings, 24 hrs	Biotin	Egg white preparation*	Galvanometer readings 24 hrs
$\gamma$ per 10 cc	cc per 10 cc		mg per 10 cc	cc per 10 cc	
0	0	93	0.0	0	93
10	0	91	0.1	0	88
30	0	76	1.0	0	53
100	0	62	10.0	0	38
30	0.00	76	0.0	2.00	100
30	0.20	85	1.0	0.20	93
30	0.40	86	1.0	0.40	96
30	0.75	87	1.0	0.75	100
30	1.00	88	1.0	1.00	100
30	2.00	88	1.0	2.00	100
100	0.00	62			
100	0.20	66			
100	0.40	62			
100	0.75	63			
100	1.00	66			
100	2.00	63			

\* Prepared by adding 5 cc of fresh egg white aseptically to 95 cc of sterile saline

transferred to a second 10 cc of sterile saline, and 1 drop of this dilute suspension used to inoculate each tube. The assay procedure has been described earlier in this paper.

**Results**—The effect of adding biotin, Tween 80, Tween 40, or oleic acid to the basal medium on growth of each of the test organisms, and some interrelationships between these factors, are shown in Table II. In the absence of biotin, 10 mg of Tween 80 support essentially maximum growth of each of these organisms. Tween 40 has slight activity for some organisms, but is relatively inactive compared to Tween 80. For *Streptococcus faecalis* and *Leuconostoc mesenteroides*, oleic acid is more effective in replacing biotin at an initial pH of 7.0 than it is at 5.5, but this relationship

is just the opposite for the three lactobacilli. With *Lactobacillus casei*, the very slight growth obtained at 48 hours is in accordance with observations of Williams and Fieger (4) that maximum stimulation with oleic acid is obtained only after 96 to 120 hours of incubation. The effect of adding Tween 40 to tubes containing oleic acid is very marked. In all cases, the response to oleic acid is very significantly increased. The results with *Lactobacillus casei* are particularly marked. That Tween 40 exerts its effects on utilization of the fatty acid, and does not merely cause more efficient utilization of traces of biotin, is shown by the fact that Tween 40 has no effect on utilization of small amounts of added biotin.

The data of Table III demonstrate that oleic acid actually replaces biotin, rather than simply accentuating the action of small amounts of biotin in the medium. The titer of a crude egg white preparation used as a source of avidin was determined against 1 m $\mu$  of biotin. Levels of 0.75 cc or greater of this dilute preparation completely prevent utilization of this amount of biotin in addition to the small amount of biotin present as a contaminant in the medium. Although increasing amounts of avidin significantly depress growth at a constant low level of oleic acid (30  $\gamma$ ), this effect does not extend to higher levels of avidin. It is probable that this effect represents nothing more than elimination of the growth-promoting effect of the small amount of biotin present as a contaminant in the medium. With higher levels of oleic acid (100  $\gamma$ ), the effect is even less marked.

#### DISCUSSION

Oleic acid is now known to be an essential growth factor for several organisms. Perhaps the first report of its essential nature for a microorganism was that of Cohen, Snyder, and Mueller (15), who showed that it was essential for growth of certain strains of *Corynebacterium diphtheriae* from small inocula. Subsequently, it has been reported as essential for *Clostridium tetani* and *Clostridium welchii* (16), for *Erysipelothrix rhusiopathiae* (12), and for an unidentified micrococcus (10). The present report demonstrates its essential nature for growth of several of the lactic acid bacteria in purified media.

The toxic action of fatty acids, especially unsaturated fatty acids, for bacteria has likewise been widely noted (2, 10, 11, 17), and although the reasons for this toxicity are not clearly understood, it is generally agreed that toxicity is observed only with the unesterified acids. Kodicek (17) observed that the inhibitory effects of oleic, linoleic, and linolenic acids for *Lactobacillus casei* could be prevented by addition of a number of compounds, such as lecithin, cholesterol, calciferol, lumisterol,  $\alpha$ -tocopherol, and, in some cases, even calcium. Saponin and sodium taurocholate were

ineffective For *Erysipelothrix rhusiopathiae*, on the other hand, the toxicity of oleic acid was prevented by both saponin and bile salts (12) Dubos and coworker (10, 11) found the Tweens to be convenient, water-soluble forms of fatty acids for use in bacterial nutrition They have shown that for some organisms the Tweens alone suffice to prevent toxicity of the fatty acids, for others, addition of materials such as serum albumin is necessary for this purpose Under the conditions used, the Tweens alone perform this function for the lactic acid bacteria studied here It seems likely that this fact could be applied to eliminate the interfering effects (1-3) which fatty substances, especially free fatty acids, have on microbiological assays for riboflavin and pantothenic acid in which these organisms are employed At present, these effects are avoided by treatment of the samples before assay to eliminate fatty substances The emulsifying and solubilizing effects of the Tweens for fat-soluble materials should greatly facilitate testing the effects of such substances on bacterial growth

Stokes *et al* (18) have shown recently that when aspartic acid is eliminated from the medium the amount of biotin required for growth of several lactic acid bacteria is greatly increased It seems likely from recent observations (19)<sup>3</sup> that the decreased requirement for biotin in the presence of aspartate results from participation of biotin in the fixation of CO<sub>2</sub> to pyruvate to yield oxalacetate, which can also arise from, or be converted to, aspartate In the presence of a product whose formation it ordinarily catalyzes, the requirement for biotin is thus greatly decreased Using media which contain ample aspartic acid, Williams and Fieger (4) have shown that oleic acid or rice oil eliminates or greatly reduces the requirement for biotin Data in the present paper extend this finding to several other lactic acid bacteria, and indicate that under these conditions an external source of biotin is unnecessary By analogy with the relationship between biotin and aspartate, it seems probable that biotin is essential for synthesis of oleic acid When both oleate and aspartate are supplied to these organisms, biotin appears unnecessary for growth Systematic elimination of other components of the complex medium used might reveal other components in whose metabolism biotin is important

Trager (5) recently reported the preparation of a fraction, FSF, from blood plasma in the presence of which biotin was non-essential for *Lactobacillus casei* In contrast to oleic acid, which was relatively ineffective in replacing biotin at an initial pH of 6.3 to 6.8, FSF was fully effective at this pH From the present data it can be seen that oleic acid, too, is effective at this pH if tested in the presence of Tween 40 Like that of FSF, the activity of oleic acid is not nullified by excess avidin These

<sup>3</sup> Eakin, R. E., and Shive, W., private communication



data would suggest that FSF contained combined oleic acid which was available to the test organism. However, FSF is reported (5) to appear in the non-saponifiable fraction of plasma, which would not be true of known combinations of oleic acid.

#### SUMMARY

Several cultures of lactic acid bacteria require oleic acid, linoleic acid, or a combined source of these materials for growth on a medium containing all of the previously recognized growth factors for these organisms. The even numbered, saturated fatty acids from  $C_6$  to  $C_{18}$  are completely inactive.

For the strain of *Lactobacillus bulgaricus* tested, oleic acid, though essential for growth, is so toxic that its growth-promoting action can be observed only within a narrow range of concentrations and pH. Addition of certain water-soluble emulsifying agents and surface tension depressants, such as Tween 40, which are inactive by themselves, render oleic acid non-toxic, and greatly extend the pH range over which activity is observed. Tween 80, a water-soluble ester of oleic acid, is an excellent, non-toxic source of oleic acid for use in culture media for these organisms.

Most of the lactic acid bacteria commonly used as assay organisms do not require oleic acid for growth on complete media. If biotin is omitted, however, oleic acid becomes essential for growth. The effectiveness of oleic acid in this respect is greatly enhanced by the simultaneous presence of Tween 40. Tween 80, in adequate amounts, has the same effect as Tween 40 plus oleic acid. The growth-promoting action of oleic acid under these conditions is not nullified by excess avidin, as is that of biotin. It is suggested that one function of biotin is to catalyze the synthesis of oleic acid, when the latter is supplied preformed in the complex medium used, biotin becomes non-essential.

*Addendum*—Since submission of this paper, two "Letters to the Editors" bearing on this subject matter have appeared. The essential nature of oleic acid for certain lactic acid bacteria has been discovered independently by Hutchings and Boggiano (20). Avelrod, Hofmann, and Daubert (21), in reporting that both oleic acid and a vaccenic acid fraction from beef tallow will replace biotin for *Lactobacillus casei*, mention that a neutral fraction from plasma which also has this action was found (in contrast to Trager's FSF) to have its activity in the saponifiable portion. This gives added support to the hypothesis, suggested above, that the activity of such neutral plasma fractions is due to active fatty acids in combined form.

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# A STUDY ON TRANSMETHYLATION WITH METHIONINE CONTAINING VARYING AMOUNTS OF DEUTERIUM IN THE METHYL GROUP

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(Received for publication, July 14, 1947)

In the course of our investigation of the conversion of deuteriomethionine to choline and creatine in the rat, we decided to investigate the effect of the concentration of deuterium in the methionine fed upon the percentage of methyl groups in choline and creatine which are derived from methionine. If a systematic study should reveal differences dependent upon the deuterium content of the compound fed, the implications for the use of deuterium as an indicator in studies of the rate of transmethylation *in vivo* would be far reaching. Accordingly, a series of experiments was undertaken in which methionine containing concentrations of approximately 80, 40, and 10 atom per cent deuterium in the methyl group, respectively, was fed, after 6 days the choline and creatine were isolated from the tissues of the rats. The deuterium content of the methyl groups of the tissue compounds was then determined. The results showed that there was a constant ratio between the atom per cent of deuterium in the methyl group of the methionine administered and the atom per cent of deuterium in the methyl groups of the choline and creatine isolated. Thus, the concentration of deuterium in the compound administered has no effect on the percentage of methyl groups in choline or creatine which have been derived from the methyl group of methionine.

## EXPERIMENTAL

In the experiments to be described, immature rats were fed diets containing 0.6 per cent methionine (Table I). However, the methionine fed had three different levels of deuterium concentration, *i.e.*, 79.1, 39.6, and 9.9 atom per cent deuterium in the methyl group respectively. The deuteriomethionine was prepared by the usual procedure (2). The food intake was carefully measured, and from this the amount of ingested methionine was calculated. 1 cc. of an aqueous vitamin solution was given daily to each rat and contained the following amounts of each vitamin in mg: thiamine hydrochloride 0.04, riboflavin 0.04, nicotinic acid 0.04, pyridoxine hydrochloride 0.04, *dl*-calcium pantothenate 0.20, choline-free ryzamin-B (3) 50.0, inositol 3.0, and *p*-aminobenzoic acid 1.0.

TABLE I  
*Composition of Basal Diet*

	<i>per cent</i>
Deuteriomethionine	0.6
Amino acid mixture*	21.3
Salt mixture†	4.0
Corn oil and fat soluble vitamins‡	1.0
Crisco	19.0
Sucrose	54.1

\* Synthetic amino acid mixture devoid of sulfur amino acids (1)

† Osborne, T. B., and Mendel, L., *J. Biol. Chem.*, **37**, 572 (1919)

‡ Fat-soluble vitamins A, D, E, and K mixed with the corn oil in the amounts described previously (1)

TABLE II  
*Platinum Analyses*

Rat No	Choline chloroplatinate	
	Pt calculated*	Pt found
	<i>per cent</i>	<i>per cent</i>
1514	31.5	32.0
1515	31.5	31.6
1516	31.6	31.4
1517	31.6	31.2
1518	31.6	31.8
1519	31.6 (N 4.5)	32.9 (N 4.5)

\* Calculated values, based on increased molecular weight due to deuterium in the molecule

TABLE III  
*Deuterium Analyses*

Rat No	Choline chloroplatinate	Creatinine K picrate
	<i>atom per cent</i>	<i>atom per cent</i>
1514	8.30 ± 0.09	2.96 ± 0.12
1515	8.21 ± 0.11	2.91 ± 0.09
1516	3.95 ± 0.11	1.28 ± 0.07
1517	3.98 ± 0.13	1.54 ± 0.08
1518	0.87 ± 0.09	0.37 ± 0.05
1519	0.93 ± 0.10	0.35 ± 0.06

It should be noted, as is indicated in Table IV, that one rat of each pair, namely Rats 1515, 1516, and 1519, developed hemorrhagic kidneys on this diet. Enlarged kidneys were observed in these three animals on the 6th day and all six rats were sacrificed immediately. Choline was isolated

from the tissues is choline chloroplatinate (Table II), and creatine as creatinine potassium picrate (2). The purity of the picrates was determined colorimetrically by the Jaffe reaction. The deuterium contents of the isolated compounds are listed in Table III.

It can be seen from the summarized data in Table IV that, in spite of the development of hemorrhagic kidneys in three rats, the percentage of methyl groups in the tissue compounds derived from methionine was approximately the same in all six animals.

TABLE IV  
Controlled Feeding Experiments for 6 Days with Three Different Levels of  
Deuterium in Ingested Methionine

Rat No and sex	Change in body weight	Deuterium in methyl group of compound fed	Amount of methionine ingested	Deuterium in methyl group of compound isolated		Per cent of methyl groups in tissue compounds derived from methionine ingested*	
				Choline	Creatine	Choline	Creatine
	gm	atom per cent	gm	atom per cent	atom per cent		
1514 ♀	57-75	79.1	0.252	12.9 ± 0.1	11.8 ± 0.5	16.3	15.6
1515 ♂†	60-74	79.1	0.210	12.8 ± 0.2	11.6 ± 0.4	16.2	14.7
1516 ♀†	59-63	39.6	0.180	6.1 ± 0.2	5.1 ± 0.3	15.5	12.9
1517 ♂	61-78	39.6	0.252	6.2 ± 0.2	6.2 ± 0.3	15.6	15.6
1518 ♀	62-72	9.9	0.198	1.35 ± 0.14	1.5 ± 0.2	13.7	14.9
1519 ♂†	62-70	9.9	0.192	1.45 ± 0.16	1.4 ± 0.2	14.6	14.1

\*  $\frac{\text{Amount of deuterium in methyl group of isolated compound}}{\text{Amount of deuterium in methyl group of methionine ingested}} \times 100$

† Enlarged kidneys observed on the 6th day, kidneys found to be hemorrhagic at autopsy.

#### SUMMARY

The concentration of deuterium in the methyl group of methionine administered to rats was varied from 10 to 80 atom per cent and was found to have no effect on the percentage of methyl groups of choline and creatine derived from methionine. Some of the animals developed hemorrhagic kidneys under the conditions of the experiment, but apparently this had no appreciable effect on the over-all rate of transmethylation.

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# THE VERATRINE ALKALOIDS

## XXXII FURTHER STUDIES WITH JERVINE

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(Received for publication, July 16, 1947)

Jervine and, in relatively smaller amount, veratramine, either as such or in the form of glycosides, are the principal bases which have been found in the alkaloid fraction of *Veratrum viride* and their further study has been of special interest. Previous work (1, 2) has shown their formulations to be respectively  $C_{27}H_{33}O_3N$  and  $C_{27}H_{33}O_2N$  and that they are secondary bases. In this respect and in a greater degree of unsaturation they present a variation of the usual category of veratrine alkaloids such as cevine, germine, and rubijervine which are tertiary bases. Recent work (3, 4) has shown that there is now no reason to believe that these tertiary bases possess other than the usual steroid structure demonstrated with the potato base, solanidine, by its dehydrogenation to Diels' hydrocarbon. The same conclusion should be possible in the case of jervine and veratramine. In veratramine, however, it is necessary to assume a rearranged angular methyl group to permit the benzenoid structure shown by its ultraviolet absorption spectrum and the three resistant double bonds indicated by the formulation. Although the formulation of jervine differs only by an O atom from that of veratramine, its absorption spectrum has presented a different picture, as is presented again in Fig. 1. In addition, the presence of at least two double bonds was shown by the earlier hydrogenation studies which led to a tetrahydrojervine (1, 5). At first, on the basis of an incomplete absorption curve (6), the double bonds were considered to be conjugated, but the more recent curve suggests other interpretations, especially that of a  $\Delta^{\alpha\beta}$ -ketonic structure or of conjugated double bonds and an isolated CO group. The present report is on further work with jervine.

The usual 3-hydroxy- $\Delta^5$  structure has been indicated by the conversion of jervine with aluminum tert-butoxide and acetone to  $\Delta^4$ -jervone. The same substance was obtained, in somewhat poorer yield although more readily purified, by heating with copper. This ketone was characterized by its oxime.  $\Delta^4$ -Jervone with aluminum isopropoxide was reduced in turn to  $\Delta^4$ -jervine. This substance was fractionally crystallized from what was undoubtedly a mixture of at least 3- $\alpha$ (OH) and 3- $\beta$ (OH) epimers, but, because of the solubility of the digtonides, the usual attempts at complete separation have been deferred. Of significance was the gradually developing, rather deep purple color given by the substance with trichloro-

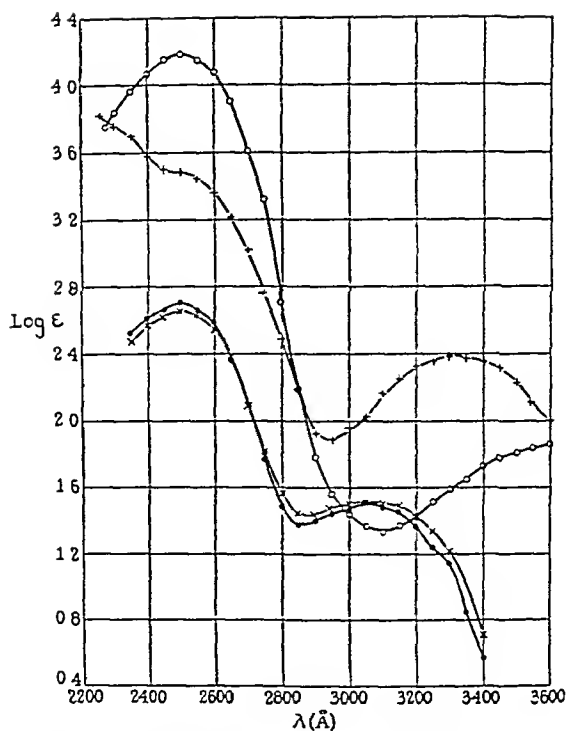


FIG 1 ○ = jervine, + = isojervine, ● = dihydrojervine, × = dihydrojervine after chromatography, all in ethanol

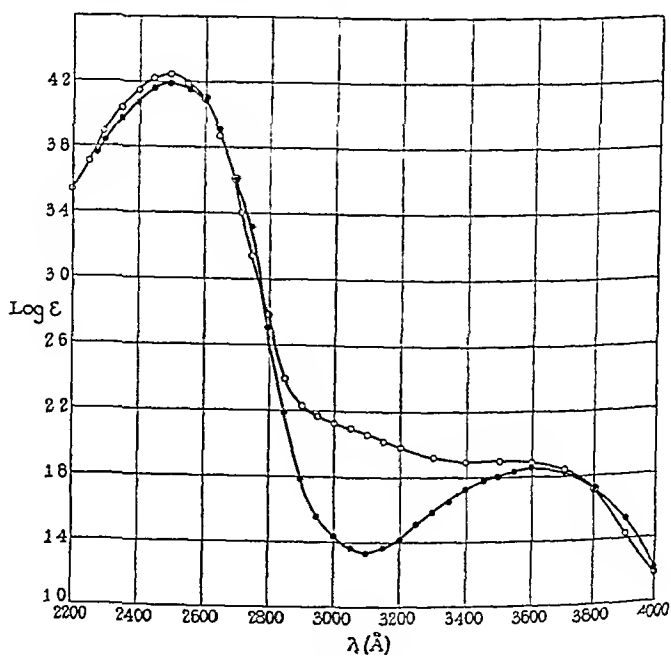


FIG 2 ○ = Δ¹-jervine, ● = jervine



acetic acid, although not as strong as in the case of the allorubijervines or allosorubijervines. In addition, the absorption spectrum of  $\Delta^4$ -jervine, as given in Fig 2, shows that the isomerization has not too greatly affected the chromophorically active feature of the molecule and the retention of a  $\Delta^{\alpha,\beta}$ -ketonic type of absorption.

The absorption spectrum of  $\Delta^4$ -jervone also has been compared with those obtained with  $\Delta^4$ -solanidone and with rubijervone, as shown in Fig 3.  $\Delta^4$ -Jervone remains, however, in general more strongly absorbing

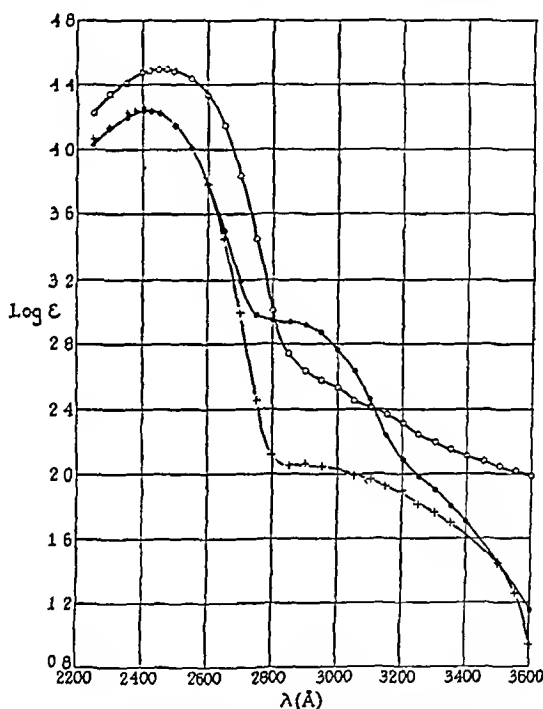


FIG 3 ○ =  $\Delta^4$ -jervone, ● = rubijervone, + = solanidone, all in ethanol

The previous hydrogenation of jervine in acetic acid solution occurs with the absorption of 2 moles of  $H_2$  and the direct formation of tetrahydrojervine (1, 5). In more recent work the process has been carried out in stages. When performed in ethanol solution, the absorption occurs very slowly and requires a number of days for apparent completion. However, the hydrogenation did not go perceptibly beyond the 1 mole stage with the formation of *dihydrojervine*, ( $[\alpha]_D^{27} = -83^\circ$ ). As with jervine itself, this derivative has yielded an *N*-acetyldihydrojervine and an *N*-acetyldihydrojervine acetate and all attempts to acylate beyond the latter stage were unsuccessful.

When the hydrogenation was resumed with dihydrojervine in acetic acid,

an additional mole of  $H_2$  was absorbed, with the formation of the above tetrahydrojervine obtained in one operation from jervine. The rotation now found was  $[\alpha]_D^{28} = -18^\circ$ . It is probable that the previously described, less soluble, so called isomer (m.p., 228–232°) was a small fraction of intermediate dihydrojervine. The tetrahydro derivative yields an *N*-acetyl tetrahydrojervine.

At this point the absorption spectra of these substances became of interest. As is shown in Fig 1, the curve obtained with dihydrojervine continued to show two peaks, the higher of which remained at  $250 m\mu$ , the lower one being considerably displaced to about  $300 m\mu$ . This suggested the persistence of a conjugated or  $\Delta^{\alpha, \beta}$ -ketonic structure, with the exception that the peak at  $250 m\mu$  appears lower than is typical of these compounds.

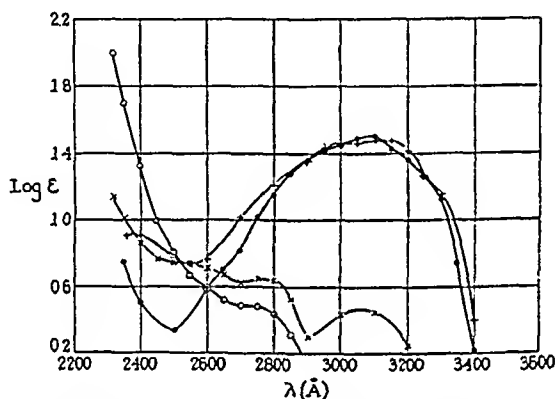


FIG 4 ○ =  $\alpha$  dihydrojervinol, ● = tetrahydrojervine, + = tetrahydrojervine after treatment with acid, × = tetrahydrojervinol, all in ethanol

The possibility was considered of a lack of homogeneity of the dihydrojervine and of its contamination if not with unchanged jervine at least with material which absorbed in the shorter wave-lengths. To test this, dihydrojervine was repeatedly recrystallized, followed by an attempt to fractionate it with  $Al_2O_3$ . An intermediate fraction recovered after such treatment, however, showed no appreciable change in the absorption, as shown in Fig 1. In addition, the same result was obtained with samples from different hydrogenation experiments. There appears, therefore, no reason to doubt that this curve is produced by dihydrojervine itself.

The curve in Fig 4, obtained with tetrahydrojervine, shows the retention of the peak at about  $310 m\mu$ , the position and intensity of which suggest carbonyl group absorption. All attempts, however, to obtain an oxime with any of these substances have been unsuccessful and jervine was recovered unchanged on attempted reaction with aluminum isopropoxide. Although we were hindered in this respect, additional evidence of the

presence of a CO group was afforded by reduction with sodium in butanol. A so called tetrahydrojervine obtained in this way from jervine has already been described (1). The further study of the substance ( $[\alpha]_D^{27} = -107^\circ$ ) has confirmed the formulation  $C_{27}H_{43}O_3N$  but indicates that its production must involve reduction of the CO group and a double bond. The curve in Fig. 4 shows the disappearance of the characteristic bands of jervine. This substance is therefore to be interpreted as  $\alpha$ -dihydrojervinol and the reason for the prefix will appear later. It yielded on acylation an *N*-acetyl- $\alpha$ -dihydrojervinol.

The result with jervine was confirmed by the reduction of tetrahydrojervine with sodium. The absorption curve obtained with the resulting *tetrahydrojervinol* is shown in Fig. 4.

In the formation of  $\alpha$ -dihydrojervinol from jervine it appears probable that the  $\Delta^5$  double bond was the one reduced, in analogy with previous experience with the  $\Delta^5$  bases such as solamine, rubijervine, and others of the group. In support of this the reduction of dihydrojervine with sodium has been found to involve now only the CO group, with the production of a substance ( $[\alpha]_D^{28} = -4^\circ$ ) isomeric with the above  $\alpha$ -dihydrojervinol. This has therefore been called  $\beta$ -dihydrojervinol. The latter on hydrogenation in acetic acid absorbs only 1 mole of  $H_2$ , with the formation of the above tetrahydrojervinol. Although  $\alpha$ -dihydrojervinol, as was to be expected, did not absorb  $H_2$  in ethanol solution, it could be hydrogenated further in acetic acid but the resulting material could not be crystallized.

The retention of a  $\Delta^{\alpha\beta}$ -ketonic type of absorption in the absorption curve of  $\Delta^4$ -jervine, while somewhat modified in that of dihydrojervine, suggests that this must involve the more resistant double bond of jervine.

If in jervine itself the conjugation originally involves also the assumed  $\Delta^5$  bond, then in the transformations to  $\Delta^4$ -jervine and dihydrojervine the resistant double bond would have to shift to become conjugated with the CO group in these substances. The exact positions of the CO group or the less reactive double bond are therefore still uncertain.

The isomerization of jervine to isojervine has already been described (7) and an attempt has since been made to interpret the change. Although veratramine superficially differs from jervine by only 1 O atom, it proved to be stable to acid. It also differs by its benzenoid type of absorption spectrum and behavior on hydrogenation (2). It appeared possible that in isojervine a similar aromatization could have occurred, since the formulation  $C_{27}H_{39}O_3N$  would permit of three double bonds as well as a CO group and another ring or double bond. Isojervine has proved resistant to all attempts to hydrogenate it in ethanol solution. When this was attempted in acetic acid, isojervine was largely recovered unchanged, although a trace was obtained of the product  $C_{27}H_{45}O_2N$  described below. The absorption spectrum curve of isojervine is given in Fig. 1. This shows a considerable

modification of the original jervine curve. However, the persistence of a somewhat lowered peak at  $250\text{ m}\mu$  is apparent, although the absorption continues to increase beyond this. An accentuation and displacement towards the short end of the assumed carbonyl absorption is also apparent. This suggests that the formation of this substance involves a possible aromatization if not simple conjugation of the three double bonds as well as retention of the CO group. An additional suggestion for this can be seen in the similarity of the curve of isojervine with that of a composite curve of jervine and veratramine.

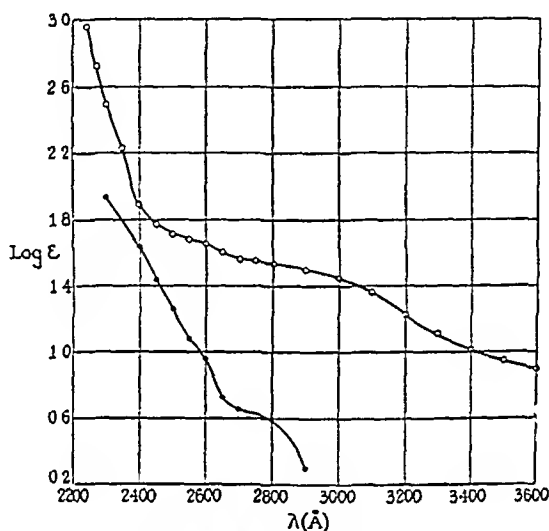


FIG 5 ○ = dihydroisojervinol, ● = hexahydrodesoxyisojervinol, in ethanol

Other differences from jervine have been noted in the behavior of isojervine. Although very stable to acid, isojervine is rapidly changed in alkaline solution. In alcoholic alkali it quickly develops a deep red color and, after short boiling, only a fraction could be recovered. On acetylation, at first *N*-acetylisojervine was obtained but the acylation then goes beyond what was found with jervine (5, 1) and readily yields the triacetyl derivative, *N*-acetylisojervine diacetate. On reduction in butanol with sodium a small yield of a substance resulted, which from the analysis could be interpreted as *dihydroisojervinol*,  $C_{27}H_{43}O_3N$  ( $[\alpha]_D^{25} = +84^\circ$ ), isomeric with the above  $\alpha$ -dihydrojervinol. The *hydrochloride* was also obtained. The absorption spectrum curve shown in Fig 5 no longer resembles that of isojervine. Although resistant to hydrogenation under the above conditions, isojervine gradually absorbed  $H_2$  in ethanol solution in the presence of excess HCl. The process required a number of days and some un-

changed material was recovered. However, the main crystalline product resulted from the absorption of 3 or 4 moles of  $H_2$  and the loss of 1 O atom. The uneventful end-absorption (Fig. 5) indicates reduction of the CO group and of double bonds. From the analytical data alone, the formulation is not certain. The substance is either a *hexahydrodesoxyisojervine*,  $C_{27}H_{45}O_2N$ , or an *octahydrodesoxyisojervine*,  $C_{27}H_{47}O_2N$ . The O atom lost is presumably either that of the CO group or the original (OH) group on carbon atom 3. The study of the substance will be continued.

A further attempt has been made to characterize the O atoms of jervine and isojervine by active H determinations. As previously shown, the presence of 2 active hydrogen atoms first reported with jervine (5) was determined at ordinary temperatures, but at  $95^\circ$  the result could be raised to 4 atoms (1). In view of the more recent work it now appears doubtful that the increased reaction at  $95^\circ$  is due to hindered OH groups. A similar difficulty has been encountered in the determinations with a number of jervine derivatives and has interfered with the use of the method in the study of these substances. It is, however, definite that the 2 active hydrogen atoms shown with jervine are due to the NH group and the (OH) group of carbon 3. Another O atom occurs in the CO group and the 3rd appears now to be of oxidic character.

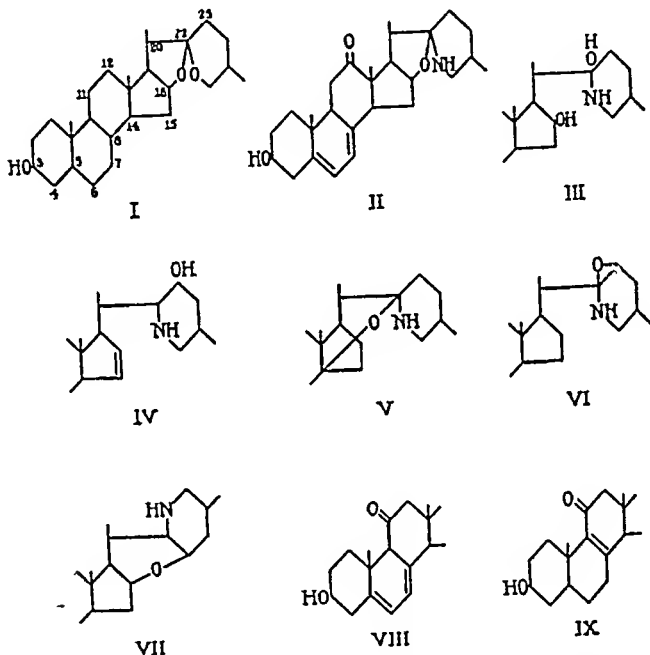
In view of the presence of the two double bonds and secondary basic pentacyclic character, such data agree with the derived formulation,  $C_{27}H_{39}O_3N$ , of jervine. It appears that on isomerization to isojervine the oxidic structure is opened, with the formation of a new OH group and a double bond. To explain such a rearrangement in acid solution, there is suggested for jervine an analogy with the spiroketal structure of the steroid sapogenins or isosapogenins (Formula I). This is presented in Formula II. However, whether such a structure could rearrange with acid and so yield the data obtained with isojervine is not certain. The first product of the action of acid could be as shown in Formula III.

If the latter does not open to form an  $\omega$ -amino ketone, it could rearrange to form the hydroxymethylethylpiperidine base, as is shown in Formula IV, and the OH on  $C^{16}$  could also be removed, with the production of a double bond initially as  $\Delta^{15,16}$  before its shift for aromatization or conjugation. A similar situation would arise if the oxidic linkage joined  $C^{14}$  with  $C^{22}$  and perhaps more readily still if between  $C^{22}$  and  $C^{23}$  (Formulas V and VI).

In a previous paper (6) a phenolic base,  $C_8H_{11}ON$ , interpreted as a possible 2-ethyl-5-methyl-3-hydroxypyridine, was reported as a dehydrogenation product of jervine. The formation of such a substance definitely requires the presence of an O atom on the basic side chain of the molecule. Another possible structure is presented in Formula VII in which an oxidic linkage joins the 2 steroid C atoms  $C^{16}$  and  $C^{23}$ . But an objection to this

interpretation is the expected resistance of such an oxidic structure to cleavage with acid

As previously mentioned, the positions of the CO group and less reactive double bond remain uncertain. If jervine itself is not a  $\Delta^{\alpha\beta}$ -ketone but in it the two double bonds are conjugated as  $\Delta^5$  and  $\Delta^7$ , as shown in Formula VIII, with the CO group at C<sup>11</sup>, a shift of the  $\Delta^7$  to  $\Delta^8$  could occur during the hydrogenation to dihydrojervine (Formula IX). A similar situation could occur if the CO group is at C<sup>12</sup>, as in Formula II



Another aspect of the general problem concerns pseudojervine. The latter has been shown to be a glucoside which, however, yields isojervine on hydrolysis (7). Since jervine is isomerized to the latter with acid, it is probable that pseudojervine is a jervine glucoside and isojervine an artifact. This has now been shown by its hydrogenation before hydrolysis. Contrary to isojervine, which resists hydrogenation, pseudojervine could be hydrogenated in ethanol as the soluble acetic acid salt with the slow absorption of 1 mole of H<sub>2</sub> as in the case of jervine itself. The resulting substance on hydrolysis yielded a base which appeared superficially indistinguishable from dihydrojervine. However, as is shown in Fig 6, the absorption curve in the short wave region differs somewhat from that of dihydrojervine. The possibility that some isomerization under the influence of the acid has occurred was suggested by the fact that dihydro

jervine itself when treated with HCl also yields material, the absorption curve of which approaches that of the hydrolysis product

A comparison of the N-acetyldihydrojervine acetates obtained from both sources has shown a somewhat similar divergence of the curves (Fig 6) It appears that a progressive change occurs under the influence of HCl and that in the experiments made this transformation probably has not proceeded to completion The conclusion, however, can be drawn that pseudojervine is a glucoside of jervine

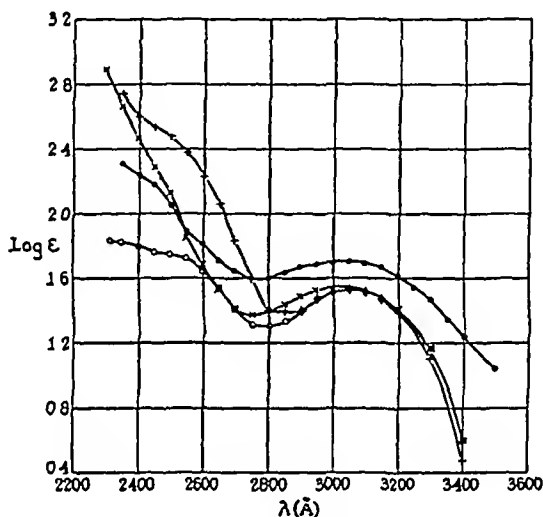


FIG 6 O = dihydrojervine after treatment with acid, ● = dihydrojervine from hydrolysis of dihydropseudojervine, + = N-acetyldihydrojervine acetate, X = N acetyldihydrojervine from acid-treated dihydrojervine, all in ethanol

#### EXPERIMENTAL

**$\Delta^4$ -Jervone**—6 gm of jervine were repeatedly boiled down with benzene to remove the  $H_2O$  of crystallization and the residue was treated with 14 gm of aluminum tert-butoxide, 200 cc of benzene, and 100 cc of acetone. The mixture was refluxed for 23 hours, then decomposed with excess dilute NaOH, and extracted with chloroform. The washed and dried extract was concentrated finally *in vacuo* to dryness. It was found better at this stage to remove unchanged jervine as the more sparingly soluble HCl salt. The residue was therefore treated with 50 cc of absolute ethanol and the suspension of crystals carefully treated with HCl (1.19 sp gr) in excess. The thick paste of the salt was collected and washed with small portions of absolute ethanol. The filtrate, after addition of excess ammonia and careful dilution, gradually crystallized. After collection with 50 per cent

ethanol, 3.06 gm of the product were obtained. The mother liquor on dilution yielded an additional 0.55 gm. It was recrystallized by solution in chloroform with partial concentration and addition of ethanol or directly from ethanol, which required a larger volume. It separated as clusters of short, narrow, blunt ended micro platelets or rods which contained solvent. After preliminary softening it melted at 193–194° (uncorrected).

$$[\alpha]_D^{25} = +28.3^\circ \quad (c = 0.95 \text{ for dry substance in 95\% ethanol})$$

Difficulty was experienced with the analysis of different samples, possibly caused by retention of solvent, even when dried at 120° and 0.2 mm.

$C_{27}H_{38}O_2N$	Calculated	C 76.54, H 8.81
Found (a)	"	76.20, " 9.11
(b)	"	76.01, " 8.94

A gradual change during the recrystallization from an original  $[\alpha]_D^{25} = +16^\circ$  to the above rotation indicated removal of a small amount of jervine.

A mixture of 1 gm of jervine and 4 gm of copper powder was gradually heated in a bath to 200° and then during 30 minutes to 290°. After sufficient cooling the mixture was then slowly sublimed at 0.1 mm, during which the temperature was finally raised to 260°. 0.75 gm of sublimate was obtained which crystallized readily from 95 per cent ethanol on seeding with the above substance. 0.34 gm resulted, which after recrystallization melted at 191–192° (uncorrected) after preliminary softening. A mixture from both sources showed no depression.

$$[\alpha]_D^{25} = +30^\circ \quad (c = 1.02 \text{ of dry substance in 95\% ethanol})$$

Found C 76.07, H 8.80

The spectrum of this substance is shown in Fig 3.

The *oxime* was prepared from a solution of the base in sufficient dilute acetic acid with a mixture of hydroxylamine hydrochloride and sodium acetate. After addition of methanol and heating, it rapidly crystallized. The mixture was then made alkaline and extracted with chloroform. The latter yielded material which crystallized from ethanol as sparingly soluble prisms melting at 287–289°.

$$C_{27}H_{38}O_2N_2 \quad \text{Calculated, C 73.92, H 8.74, found, C 74.00, H 8.97}$$

$\Delta^4$ -Jervine—0.42 gm of  $\Delta^4$ -jervone in benzene was concentrated to dryness *in vacuo* to remove any solvent of crystallization and then treated with aluminum isopropoxide made from 0.25 gm of Al in about 30 cc of isopropanol. The mixture was very slowly distilled at the rate of about a drop a minute, with occasional replacement of isopropanol until no further test for acetone with nitroprusside was obtained. After about 4 hours, the concentration was continued to small bulk. The mixture was treated



with excess dilute  $\text{NaOH}$  and extracted with chloroform. The latter, after washing and drying, was concentrated and the solvent replaced by ethanol. The concentrated solution on careful dilution gradually crystallized on standing and was collected the next day with 50 per cent ethanol. 0.2 gm was obtained. The mother liquor on further careful dilution yielded an additional 75 mg.

The first crop was recrystallized by solution in ethanol and concentration to about 2.5 cc and then carefully treated with an equal volume of  $\text{H}_2\text{O}$ . On seeding, it gradually separated as aggregates of needles or pointed blades and was collected with 50 per cent ethanol. It sintered at about  $185^\circ$  and gradually melted at  $203\text{--}207^\circ$  but not completely below  $211^\circ$ .

The substance contained solvent and was dried at  $110^\circ$  and 0.2 mm.

C $\text{H}_{13}\text{O}_2\text{N} \cdot \text{H}_2\text{O}$	Calculated, H 4.06, found, H 3.70
C $\text{H}_{13}\text{O}_2\text{N}$	Calculated C 76.18, H 9.24
	Found (a) " 75.77, " 9.23
	(b) " 75.84, " 9.59

Since the substance could be a mixture possibly of 3- $\alpha$  and 3- $\beta$  epimers and did not yield a sparingly soluble digitonide, no attempt at further separation was made. When 1 mg of the substance was treated with 3 small drops of 90 per cent trichloroacetic acid, it gradually developed a somewhat dirty but deep red purple color. This persisted but varied somewhat with different fractions and was not as intense as that given by epiallorubijervine. The ultraviolet absorption curve is shown in Fig. 2. Curves obtained with several different fractions and preparations were almost indistinguishable.

*Dihydrojervine*.—A suspension of 10.5 gm of jervine in absolute ethanol was hydrogenated at first with 0.2 gm of platinum oxide catalyst. The absorption of  $\text{H}_2$  proceeded gradually and since, after 2 days, only a fraction of a mole had been absorbed, an additional 0.2 gm of catalyst was added. After about 5 days in all, absorption had practically stopped, with a total for the substance of about 1 mole. The catalyst, which remained colloidal, was coagulated by addition of acetic acid, followed by final clearing with norit. The filtrate, after concentration and dilution, was made alkaline and extracted with chloroform. This extract in turn was concentrated to dryness and the residue, on treatment with methanol, crystallized. Two successive fractions were obtained of indistinguishable character and amounted to 8.7 gm. Additional fractions were not investigated further.

From methanol it crystallized as flat, largely four-sided prisms which contained solvent. On rapid heating, it sintered above  $145^\circ$ , melted with loss of solvent at about  $160^\circ$ , quickly resolidified, and again melted at  $250\text{--}251^\circ$  (uncorrected). From dilute ethanol it similarly crystallized with

solvent and, after preliminary sintering above 135–140°, remained crystalline to about 235°. From acetone it separated solvent-free as platelets and flat needles which melted at 248–251°

$[\alpha]_D^{25} = -82^\circ$ (c = 0.98 of dried substance in 95% ethanol)			
C <sub>17</sub> H <sub>11</sub> O <sub>3</sub> N	Calculated	C 75.82, H 9.67	
	Found (a)	" 75.58, " 9.72	
	(b)	" 75.79, " 9.46	

Because of the character of the ultraviolet absorption spectrum a further attempt at possible purification was made. Following two recrystallizations from methanol and a third from acetone, the melting point was unchanged. About 0.18 gm was dissolved in 20 cc of benzene and chromatographed through a 15 × 12 cm column of Al<sub>2</sub>O<sub>3</sub>. Since 100 cc of benzene failed to elute any substance, this was followed by 2 per cent methanol in benzene. After an initial 40 cc, four succeeding fractions were obtained, a first fraction of 10 cc which contained roughly 10 mg, a second of 5 cc with 80 mg, a third of 5 cc with 62 mg, and a fourth of 10 cc which removed the remainder. The third fraction was recrystallized from benzene and dried at 100° for 12 hours for the absorption spectrum shown in Fig. 1. This is indistinguishable from the curve obtained before fractionation.

The dried material from benzene under the microscope after preliminary enlargement of crystals melted sharply at 268–269°. However, when this sample was recrystallized from acetone, it melted at 248–252°, as in previous cases.

Found C 75.89, H 9.68

*Hydrolysis of Dihydropseudojervine*—0.3 gm of pseudojervine in 3 to 4 cc of 95 per cent ethanol and 0.1 cc of acetic acid were hydrogenated with 50 mg of platinum oxide catalyst. As in the case of jervine itself, the hydrogenation proceeded very gradually and after about 3 days was practically completed. The absorption in excess of catalyst requirements was 11 cc or about 1 mole. The catalyst was largely colloidal and was removed with norit after faint acidification with HCl. The filtrate was concentrated *in vacuo* to remove alcohol, during which crystallization of the salt of the hydrogenated glycosidal base occurred. The mixture was directly treated with 60 cc of 2 per cent aqueous HCl and refluxed for 1 hour. The HCl salt of the base crystallized and was collected with dilute HCl. A solution of the salt in hot ethanol was concentrated to a few cc, diluted, and treated with ammonia. The base separated as a crystalline powder and 96 mg were collected with dilute ethanol. On recrystallization from 50 per cent ethanol, the material separated as long narrow micro platelets or needles which contained solvent. After preliminary melting

at about 135–140°, the substance crystallized again and then melted at 246–249°. From acetone it separated solvent-free and melted sharply at 251–252°. When mixed with dihydrojervine, the melting point was not changed

$[\alpha]_D = -83^\circ$  ( $c = 0.99$  of dried substance in 95% ethanol)  
 $C_{21}H_{41}O_5N$  Calculated, C 75.82, H 9.67, found, C 75.55, H 9.57

The absorption spectrum is shown in Fig. 6

For comparison with this material, 0.1 gm. of dihydrojervine was heated in 4 cc. of 3.85 per cent HCl in methanol at 100° for 23 hours. Because of the suspension of crystals which remained throughout the heating, the mixture was occasionally shaken. The collected crystals weighed 60 mg. An additional 26 mg. were obtained from the mother liquor with ether. The base was recovered from both fractions in 50 per cent ethanol with ammonia. After preliminary sintering with solvent loss, it melted at 244–249°.

Found (a) C 75.83, H 9.58, (b) C 75.70, H 9.67  
 $[\alpha]_D = -83^\circ$  ( $c = 0.89$  of dried substance in 95% ethanol)

The absorption spectrum of this substance is shown in Fig. 6

*N-Acetyldihydrojervine*—0.3 gm. of dihydrojervine dissolved readily on heating in a few cc. of acetic anhydride. After boiling a few minutes, the reagent was removed *in vacuo*. The residue was dissolved in ethanol, treated with excess dilute NaOH, and boiled a short time for saponification. On dilution it crystallized. The mixture was extracted with chloroform and the extract washed with dilute acid and water. After concentrating, the residue crystallized readily from 95 per cent ethanol and yielded 0.27 gm. It formed almost square ended or hexagonal platelets which contained solvent. It melted at 157–159° and then crystallized on further heating and again melted at 256–259° (uncorrected).

For analysis it was dried at 100° and 0.2 mm.

$C_{23}H_{43}O_4N$  Calculated, C 74.14, H 9.23, found, C 74.45, H 9.23

*N-Acetyldihydrojervine Acetate*—Dihydrojervine was refluxed for 3 hours in acetic anhydride. The product separated from dilute ethanol as broad leaflets or clusters of narrow leaflets or flat needles. After recrystallization it melted at 210–212°.

$C_{31}H_{46}O_6N$  Calculated, C 72.75, H 8.87, found, C 73.00, H 8.72

A substance indistinguishable in form from the above resulted by acetylation of the previously described material recovered from the action of HCl in methanol on dihydrojervine. After recrystallization from dilute ethanol it melted largely at 214–216° but crystals persisted till 222°.

although this is slightly higher than the above melting point No depression was noted with a mixture of the two substances

Found C 72.90, H 8.77

The ultraviolet absorption spectra of the two substances are recorded in Fig. 6 which shows an increasing difference towards the short end

*Tetrahydrojervine*—2.2 gm. of dihydrojervine were hydrogenated in acetic acid with 0.1 gm. of platinum oxide catalyst. The absorption was gradual but was completed within 18 hours, during which the catalyst had coagulated. The filtrate was concentrated, diluted with water, and then made alkaline and extracted with chloroform. After concentration of the extract the chloroform was finally replaced with acetone. The product separated as small almost rectangular platelets or flat prisms.

For recrystallization its solution in methanol was concentrated and after addition of acetone again concentrated to remove methanol. It then melted at 216–221° after preliminary sintering above 213°.

$[\alpha]_D^{25} = -18^\circ$ ( $c = 0.95$ in 95% ethanol)			
$C_{27}H_{45}O_2N$	Calculated	C 75.46, H 10.10	
	Found (a)	" 75.68, " 9.94	
	(b)	" 75.23, " 9.98	

The previously described tetrahydrojervine, m.p. (uncorrected) 210–212°, which was prepared by a one-step hydrogenation process has since been found to show  $[\alpha]_D^{25} = -23^\circ$  ( $c = 1.08$  in 95 per cent ethanol).

When this substance was treated at room temperature with methanol saturated with HCl at 0° or refluxed for 19 hours in 100 parts of 2 per cent HCl in 50 per cent ethanol, it was recovered practically unchanged. The spectra of these two substances are shown in Fig. 4.

*N-Acetyltetrahydrojervine*—Tetrahydrojervine was dissolved in warm acetic anhydride and the excess reagent was removed *in vacuo*. The residue was boiled a few minutes in methanol and dilute NaOH to saponify any possible ester. After dilution the product was extracted with chloroform which was washed in turn with dilute  $H_2SO_4$  and water and dried. Upon removal of solvent the neutral product crystallized slowly from dilute ethanol as flat needles which melted at 266–269°.

$C_{29}H_{45}O_4N$  Calculated, C 73.83, H 9.62, found, C 74.03, H 9.42

*$\alpha$ -Dihydrojervinol*—The reduction of jervine with sodium in butanol was repeated as previously reported, but the product was extracted with chloroform. The yield was roughly one-half the theoretical. When reduction in ethanol was attempted, a large fraction of jervine was recovered. For recrystallization the concentrated solution in methanol was treated with

acetone. It separated solvent-free and melted at 223–225° but the melting point depended on the heating rate.

$[\alpha]_D^{25} = -107^\circ$  ( $c = 0.91$  in 95% ethanol)  
 $C_{27}H_{44}O_2N$  Calculated, C 75.46, H 10.10, found, C 75.55, H 10.03

The ultraviolet absorption spectrum of this substance is shown in Fig. 4.

*N-Acetyl- $\alpha$ -dihydrojervinol*—After short boiling of the base with acetic anhydride, followed by removal of excess reagent and by heating of the residue with dilute alcoholic alkali for saponification, the neutral material was extracted with chloroform. The substance separated from dilute ethanol as short, flat, square ended needles or rods which melted at 150–151° after slight preliminary sintering.

$C_{27}H_{44}O_2N$  Calculated, C 73.83, H 9.62, found, C 73.76, H 9.65

*$\beta$ -Dihydrojervinol*—1.25 gm. of dihydrojervine were reduced in 125 cc. of butanol with 7 gm. of sodium. The chloroform extract of the diluted reaction mixture after concentration yielded a residue which crystallized from acetone. The yield was 0.88 gm. It is rather sparingly soluble in methanol from which it separated as small prisms or platelets melting at 286–289° after slight preliminary sintering.

$[\alpha]_D^{25} = -4^\circ$  ( $c = 1.07$  in 97% ethanol)  
 $C_{27}H_{44}O_2N$  Calculated, C 75.46, H 10.10, found, C 75.12, H 9.97

*Tetrahydrojervinol*—Tetrahydrojervine was reduced in butanol with sodium and the reaction product was extracted from the diluted mixture with chloroform. The substance crystallized from acetone as a powder of micro prisms or hexagonal platelets which melted at 293–296° after preliminary sintering.

$[\alpha]_D^{25} = +48.5^\circ$  ( $c = 1.05$  in 95% ethanol)  
 $C_{27}H_{46}O_2N$  Calculated, C 75.11, H 10.51, found, C 75.06, H 10.46

0.1 gm. of  $\beta$ -dihydrojervinol was hydrogenated in acetic acid with 50 mg. of catalyst. The catalyst gradually coagulated after the absorption of about 1 mole of  $H_2$ . 77 mg. of product were obtained, indistinguishable in properties from the above substance. It melted at 294–296° after preliminary sintering.

$[\alpha]_D^{25} = +48.5^\circ$  ( $c = 1.09$  in 95% ethanol)  
 Found C 74.85, H 10.37

The ultraviolet absorption spectrum is shown in Fig. 4.

*N-Acetylisojervine*—0.2 gm. of isojervine (from acetone) was dissolved in a little ethanol and dried *in vacuo* to remove all solvent. On treatment with 2 cc. of acetic anhydride and gentle warming, but not above 50°, it

it gradually dissolved within 5 minutes After decomposition with water the resinous mass crystallized on standing The collected material when recrystallized from diluted ethanol formed delicate needles which melted at 202–203° after slight preliminary softening

$C_{27}H_{41}O_4N$  Calculated, C 74.47, H 8.84, found, C 74.28, H 8.85

*Triacetylisojervine*—When isojervine was boiled in excess acetic anhydride for 1 hour, the reaction product, recovered as in the previous case, crystallized from dilute ethanol as micro platelets or rods which melted at 192–193°

The same substance resulted after boiling with the reagent for 8 hours

$C_{33}H_{45}O_6N$	Calculated	C 71.82, H 8.23
	Found (a)	" 71.83, " 8.26
	(b)	" 71.80, " 8.16

*Dihydroisojervinol*—0.3 gm of isojervine was concentrated *in vacuo* in dry butanol to remove acetone of crystallization and the residue, after solution in 20 cc of butanol, was reduced at the boiling point with 1 gm of sodium The first contact of the latter developed at once a deep red color which rapidly decolorized The cooled mixture was diluted and extracted with benzene The washed and dried extract yielded on concentration a resin which did not crystallize directly It was treated in methanol solution with sufficient HCl and, on addition of ether, the HCl salt crystallized The peroxides in the ether caused the mother liquor to deepen in color to a brown-orange 58 mg of the salt were collected with methanol-ether After solution in methanol and concentration to 1 cc, it separated as micro platelets or flat needles which sintered above 270° and lost all crystalline form at 315–320°

$C_{27}H_{43}O_3N \cdot HCl$	Calculated	C 69.56, H 9.52
	Found (a)	" 69.31, " 9.45
	(b)	" 69.68, " 9.62

The base liberated from the salt crystallized from dilute ethanol as leaflets which contained  $H_2O$  of crystallization Under the microscope the substance loses its double refraction at 127–130° and is melted at 135–140° For analysis it was dried at 110° and 0.2 mm

$C_{27}H_{43}O_3N \cdot H_2O$  Calculated,  $H_2O$  4.02, found,  $H_2O$  3.90  
 $C_{27}H_{43}O_3N$  Calculated, C 75.46, H 10.10, found, C 75.20, H 9.90  
 $[\alpha]_D^{25} = +84^\circ$  ( $c = 0.64$  of dry substance in 95% ethanol)

For the absorption spectrum see Fig 5

*Hexahydrodesoxyisojervine*—After unsuccessful attempts to hydrogenate isojervine dissolved in ethanol or acetic acid, the following conditions in the presence of HCl were used

0.2 gm was concentrated in ethanol to about 5 cc to remove acetone of crystallization and then treated with 0.2 cc of HCl (1.19 sp gr) and hydrogenated with 50 mg of platinum oxide catalyst. The absorption proceeded gradually beyond the catalyst requirements but continued for about 3 to 4 days. The filtrate with washings from catalyst was treated with excess ammonia and concentrated. On careful dilution it crystallized. More ammonia was added and the material was extracted with chloroform. The latter on concentration did not yield isojervine on seeding. The chloroform was then replaced by ethanol and the solution on careful dilution with H<sub>2</sub>O yielded micro prisms which were collected with 85 per cent ethanol. The yield was 58 mg. Further dilution yielded an additional 20 mg.

In a larger run with 4 gm of isojervine in ethanol with 4 cc of HCl and 0.2 gm of catalyst, an additional 0.2 gm of catalyst and 4 cc of HCl were added after 24 hours. Absorption of H<sub>2</sub> in this case proceeded for about 5 days, during which the apparent absorption by the substance approximated 3 to 4 moles. However, after extraction of the base with chloroform the extract yielded 1.0 gm of unchanged base, which separated with solvent. The filtrate, after removal of chloroform with ethanol and slight dilution, yielded 1.12 gm.

The substance separates as aggregates of micro prisms which contain solvent and melt at 163–165° after preliminary sintering. From acetone it also separates with solvent. The melting point was occasionally affected by the conditions of crystallization, the substance only partly melted or softened at 165° and then melted at 218–220°. For the solvent-free substance  $[\alpha]_D^{20} = +26^\circ$  ( $c = 0.73$  in 95 per cent ethanol). For analysis it was dried at 110° and 0.2 mm.

C <sub>27</sub> H <sub>47</sub> O <sub>3</sub> N	Calculated	C 77.63, H 11.35
C <sub>27</sub> H <sub>45</sub> O <sub>3</sub> N	"	" 78.01, " 10.92
	Found (a)	" 77.56, " 10.97
	(b)	" 77.82, " 10.95

The absorption spectrum curve is shown in Fig. 5

#### SUMMARY

Jervine, the secondary veratrine base of formula C<sub>27</sub>H<sub>39</sub>O<sub>3</sub>N, has been found to behave in its transformations as a 3-hydroxy- $\Delta^5$ -steroid base. From the study of the derivatives formed by its progressive hydrogenation and reduction with sodium, it must be a doubly unsaturated  $\Delta^{\alpha\beta}$ -keto derivative or one in which the double bonds are conjugated. The 3rd oxygen atom is presumably oxidic and is associated with the basic side chain of the molecule. A general tentative interpretation of the structure of the alkaloid has been made. This has been aided by a similar study of

isojervine, which results from the isomerization of jervine by acid. A number of derivatives of the alkaloid are described with the interpretation of their ultraviolet absorption spectra.

All analytical work was performed by Mr. D. Rigakos of this laboratory.

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# THE FLUORESCENT CONDENSATION PRODUCT OF N<sup>1</sup>-METHYLNICOTINAMIDE AND ACETONE

## IV A RAPID METHOD FOR THE DETERMINATION OF THE PYRIDINE NUCLEOTIDES IN ANIMAL TISSUES THE COENZYME CONTENT OF RAT TISSUES\*

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(Received for publication, July 8, 1947)

In the previous paper of this series the application of the fluorometric alkali-acetone condensation procedure to the determination of the pyridine nucleotides in blood was described (1). The attempts to apply the method used for blood to tissues gave low values compared with those obtained by previous workers using microbiological methods (2-5). These low values were due to losses, presumably because of either the failure to inactivate the cellular nucleotidases (6, 7) or to the hydrolytic effect of the trichloroacetic acid employed.

In an attempt to overcome this difficulty it was found that the use of nicotinamide as a specific inhibitor of the nucleotidases (8) was entirely effective, and did not in any way interfere with either the fluorometric procedure or the growth of *Hemophilus parainfluenzae* used for the microbiological determination (9). Therefore, in the method described below the excised tissue is immediately immersed in a solution containing nicotinamide and  $\text{Ce}(\text{SO}_4)_2$ . After suitable extraction, dilution, and filtration an aliquot is analyzed by the fluorometric method previously described (1).

Since in tissues the reduced nucleotides may amount to 40 per cent of the total (10), it is necessary to oxidize them, for which purpose ferricyanide has been used (9). But ferricyanide was found to interfere appreciably with the formation of the fluorescent derivative  $\text{Ce}(\text{SO}_4)_2$ , on the other hand, which is an even more efficient oxidizing agent, was found to have no

\* Grants in aid of this investigation from the United States Public Health Service, the Nutrition Foundation, Inc., the John and Mary R. Markle Foundation, and the Duke University Research Council are gratefully acknowledged. The diphosphopyridine nucleotide (DPN) used in the study was obtained through the courtesy of Dr. J. A. Aeschlimann of Hoffmann-La Roche, Inc.

† The data contained in this paper were taken from a thesis to be presented by Jean Robinson in partial fulfillment of the requirements for the degree of Master of Arts in the Graduate School of Duke University.

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effect on the analytic results obtained both by the fluorometric and the microbiological procedures used in this study

Extensive data are also given on the coenzyme content of rat liver, kidney, and muscle as determined by the new method. These values are in agreement with previously published data on the total nicotinic acid content and indicate that substantially all of the niacin of these tissues exists in the form of pyridine nucleotides

### Procedure

In the work described below all experiments were carried out on rat tissues. Both male and female rats of the Osborne-Mendel strain, weighing between 100 and 250 gm, were used

*Extraction of Tissues*—The procedure which gave the best and most consistent results was as follows. The animal is killed by decapitation. A whole liver lobe, a whole kidney with the capsule carefully removed, or the large portion of a single skeletal muscle (samples of 500 to 1800 mg) is rapidly excised and *immersed completely* in previously weighed 5 to 10 ml. beakers containing 4 to 7 ml. of 2 per cent nicotinamide and 15 to 20 mg of  $\text{Ce}(\text{SO}_4)_2$ .

Once the tissues are immersed, the subsequent weighing and further manipulation can be carried out at leisure, since there is no appreciable loss of tissue coenzymes for at least 4 hours at room temperature. The weighed contents of the beakers are transferred to the small cup of the Waring blender<sup>1</sup> with 10 to 15 ml. of 2 per cent nicotinamide without  $\text{Ce}(\text{SO}_4)_2$ . The tissue is ground for 30 to 120 seconds to produce a smooth homogenate. Satisfactory results can also be obtained by grinding with sand in a small mortar. The homogenate is diluted with nicotinamide solution to 50 to 100 ml. The mixture is then filtered through a No. 30 Whatman paper (1). The filtrates, which are somewhat opalescent for liver and kidney, are stable for at least 48 hours if kept refrigerated.

Appropriate dilutions are made with distilled water to suit the sensitivity of the fluorometer to be used. In this laboratory a Coleman model 12A fluorometer was used in a range of 0.125 to 1  $\gamma$  of DPN. The procedure for the development of fluorescence is exactly that used for the determination of pyridine nucleotides in blood (1). The relation of the recovery values for added DPN and N<sup>1</sup>-methylnicotinamide is constant, as previously reported, and, therefore, added N<sup>1</sup>-methylnicotinamide may be used as an internal standard. The necessity for the use of an internal standard is determined largely by the degree of dilution of the tissue extract. With the use of a more sensitive instrument one separate determination on a

<sup>1</sup> The 25 ml. metal cup now available is satisfactory for this purpose. In this work a specially designed micro cup was employed.

suitable N<sup>1</sup>-methylnicotinamide or DPN standard in a given series of analyses should be sufficient. This can be determined in individual cases.

Briefly the fluorometric analysis consists of the following steps. To each of three cuvettes is transferred 0.50 ml of extract (0.3 to 1  $\gamma$  of DPN), 0.5 ml of water is added to Tube 1, and 1.0 ml is added to Tube 2 (the blank). Then 0.5 ml of the solution containing standard (0.25  $\gamma$  of DPN) is added to Tube 3, 0.5 ml of acetone is added to Tubes 1 and 3 and these are mixed. To all tubes is now added 0.2 ml of 6 N NaOH, they are mixed and allowed to stand for 5 minutes, after which 0.3 ml of 6 N HCl is added and the tubes heated for 2 minutes in a boiling water bath. The tubes are cooled and 1 ml of 20 per cent KH<sub>2</sub>PO<sub>4</sub> is added, the contents are diluted to an appropriate volume and the fluorescence read with B<sub>1</sub> filters.

#### RESULTS AND DISCUSSION

In developing the above method the validity of the values obtained was tested and checked by comparison with those obtained by two microbiological methods: the growth assay with *Hemophilus parainfluenzae*, which is specific for the pyridine nucleotides (11), and also the assay with *Lactobacillus arabinosus* by which the total nicotinic acid content of the tissues (12) is determined. In the case of the *Hemophilus parainfluenzae* assay it was feasible to employ the identical nicotinamide extracts prepared as above for the fluorometric analysis (Table I), whereas in the *Lactobacillus arabinosus* assay (Table II) it was obviously not possible to use the extracts containing the large excess of nicotinamide, and it was necessary to resort to the simultaneous analysis of another portion of the tissue taken from the same animal and extracted by autoclaving in 1 N HCl, as recommended by Krehl and his coworkers (12).

The summary of results in Table I shows a substantial agreement of the range of the values and of their respective means obtained by the proposed fluorometric technique and the specific *Hemophilus parainfluenzae* assay in rat tissues. Not shown in Table I, but of great relevance to the problem of the determination of the pyridine nucleotides in the tissues, is the question of the duplicability of the values obtained by the two methods.

The average percentage difference between duplicate pairs in the fluorometric procedure (twelve determinations) was 0.9 per cent (range 0 to 4 per cent). In the microbiological assay (twelve determinations) the average percentage difference between duplicate pairs was 7 per cent (range 0 to 13 per cent). In six microbiological assays in which the DPN content of each of the tissues was calculated from three duplicate determinations, each at a different dilution level of the extract, the average variation of the values from their mean was 9 per cent (range 2 to 30 per cent).

In addition to the wider range of values obtained with the microbiological

procedure, which is inherent in most biological methods, the daily response of *Hemophilus parainfluenzae* to identical levels of DPN was extremely erratic. From our experience it is apparent that optimum conditions for cultivation and growth of this organism are yet to be established.

TABLE I

Summary of Data on Pyridine Nucleotide Content of Rat Tissue, Comparison of Values Obtained by Fluorometric Analyses and Specific Assay with *Hemophilus parainfluenzae*\*

The results are given in terms of micrograms of DPN per gm. of fresh tissue

	Liver, 12 analyses		Kidney, 11 analyses		Muscle, 9 analyses	
	Fluorometric	<i>H. parainfluenzae</i>	Fluorometric	<i>H. parainfluenzae</i>	Fluorometric	<i>H. parainfluenzae</i>
Range	647-1290	756-1322	394-1045	452-955	372-655	386-520
Mean $\pm$ s.d.	1006 $\pm$ 227	1036 $\pm$ 189	725 $\pm$ 228	759 $\pm$ 168	492 $\pm$ 76	454 $\pm$ 34

\* The fluorometric and microbiological assays were carried out on the same extracts of the tissues

TABLE II

Summary of Data on Pyridine Nucleotide Content of Rat Tissue As Calculated from Total Niacin Determined by *Lactobacillus arabinosus* Assay and Compared with Results Obtained by Fluorometric Method\*

The results are given in terms of micrograms of DPN per gm. of fresh tissue

	Liver, 12 analyses		Kidney, 11 analyses		Muscle, 9 analyses	
	Fluorometric	<i>L. arabinosus</i>	Fluorometric	<i>L. arabinosus</i>	Fluorometric	<i>L. arabinosus</i>
Range	720-1240	650-1320	650-1080	540-1160	440-555	385-545
Mean $\pm$ s.d.	925 $\pm$ 173	890 $\pm$ 151	810 $\pm$ 113	785 $\pm$ 119	490 $\pm$ 43	475 $\pm$ 60
	945†		725†		465†	

\* The fluorometric and microbiological data represent analyses on separate liver lobes, halves of a kidney, and opposite gastrocnemius muscles in the same animal.

† Mean DPN values calculated from the data on the total niacin content, as determined colorimetrically by Handler and Dann (5).

In addition to the specific *Hemophilus parainfluenzae* assay it was decided to verify the results obtained by the fluorometric procedure by determining the total nicotinic acid content of the tissue with the growth of *Lactobacillus arabinosus* (12). A summary of the data obtained in this comparison is shown in Table II. In spite of the fact that the analyses by the two techniques were by necessity carried out in separate samples of tissue, the agreement in the range of values, standard deviation, and means is remarkably

good This is all the more noteworthy inasmuch as the *L. arabinosus* values are known to represent the total niacin content of the tissues, whereas the acetone condensation method is known to give fluorescent derivatives only with the quarternary nitrogen derivatives of nicotinamide Since of these latter compounds only N<sup>1</sup>-methylnicotinamide and the pyridine nucleotide coenzymes have been identified so far in the animal organism, and since the former (N<sup>1</sup>-methylnicotinamide) does not produce growth with *L. arabinosus*, it can be deduced that all of the niacin present in the rat tissues studied is in the form of the pyridine nucleotides This deduction is further supported by the agreement with the values of total niacin of rat tissues obtained by the use of a colorimetric chemical method by Handler and Dann (5) in 1941, as shown in Table II In the same publication these workers showed that, while the total nicotinic acid content as determined chemically agreed with those calculated from the coenzyme values obtained with *Hemophilus parainfluenzae* in the case of rat kidney and muscle, the

TABLE III

*Summary of Data on Pyridine Nucleotide Content of Rat Tissue*

Compilation of all data obtained by the fluorometric technique Results in terms of micrograms of DPN per gm of fresh tissue

	Liver 105 analyses	Kidney 82 analyses	Muscle 65 analyses
Mean	887	743	432
Total range	490-1290	395-1240	240-655
Range of $\frac{2}{3}$ values	700-1100	570- 920	340-540

total niacin of the liver exceeded the nucleotide niacin by about 60 per cent, which difference they designated "free nicotinic acid" This discrepancy is most probably due to the partial breakdown of the nucleotides during the process of extraction with the trichloroacetic acid employed by these workers Subsequently, Handler and Klein (1942) (6) showed that rat liver has a more active nucleotidase system than either kidney or muscle and demonstrated that it could be inhibited with nicotinamide

In addition to the comparative data shown in Tables I and II a large number of analyses of the three rat tissues were carried out by the fluorometric method and a summary of these is given in Table III The values for standard deviation were not calculated, but the range of two-thirds of all values is given as obtained from a scatter diagram The range of values is wider and the means are lower than those in Tables I and II This is undoubtedly due to the larger number of animals represented and also to variation in their nutrition While some of them were maintained for several weeks on standard Purina dog chow, others were used as they came

directly from the breeding colony where they were kept on a mixed diet. The effect of experimental dietary restrictions and modifications upon the coenzyme content of rat tissues has not yet been studied by this method.

A summary of the differences in coenzyme content between different lobes of the liver, the two kidneys, and the two gastrocnemius muscles in the same animals is given in Table IV. The magnitude of variations as shown in these figures should be useful in the interpretation of other experimental data in rats.

The following technical details of the method deserve special mention. In removing tissues for analyses it was found essential to minimize the area of cut surface on which the maximal destruction of coenzyme occurs. In this study the highest values were invariably obtained when whole lobes of liver and whole kidneys rather than when pieces or slices were removed.

TABLE IV  
*Difference in Pyridine Nucleotide Content of Pieces of Same Tissue of a Rat*  
Expressed as percentage difference from the mean of the two values

	2 liver lobes, 29 pairs	2 kidneys 23 pairs	Opposite gastrocnemius muscle, 17 pairs
Range	0-31	0-37	3-44
Average difference $\pm$ s.d.	10 $\pm$ 7.8	9 $\pm$ 7.7	18 $\pm$ 13.3

It is, therefore, suggested that whenever the analysis of tissue slices is desirable losses may be prevented by slicing the tissues while they are kept submerged in nicotinamide solution. It will be noted that the preparation of tissue extracts for analyses does not involve deproteinization, as in the case of blood (1), since it was found that in the dilutions employed the presence of protein did not affect the intensity of fluorescence. If for some reason deproteinization is desirable, trichloroacetic acid may be used in conjunction with the nicotinamide, provided the pH of the extract does not fall below 1.6, since our data indicate that at pH 1.6 and below hydrolysis of the coenzymes occurs.

The advantages of the above method are as follows. The use of nicotinamide in the extraction procedure results in less loss of pyridine nucleotides than in any other commonly employed procedure. We are not aware of any known way of determining the absolute values without any loss whatsoever. Also, with this method of extraction the necessity for excessive speed in manipulation is eliminated, and thus it becomes possible to prepare and handle a larger series of tissue extracts. Once the extraction is completed, analysis of a set of six extracts, in duplicate, can be completed in 1 hour by an experienced analyst.

## SUMMARY

An adaptation of the fluorimetric acetone condensation method to the determination of the pyridine nucleotide coenzymes in animal tissues is described. The rapid destruction of the coenzymes is prevented by the use of nicotinamide in the extraction medium. The validity of this method was established by substantial agreement with values obtained by the microbiologic assay with *Hemophilus parainfluenzae*, specific for the pyridine nucleotides. Data on the pyridine nucleotide coenzyme content of rat liver, kidney, and muscle are given. The comparison with the values for total niacin as determined by assay with *Lactobacillus arabinosus* and colorimetrically (by previous workers) shows that all of the niacin in these tissues is present in the form of the nucleotides.

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# INTERRELATIONS OF PHENYLALANINE AND TYROSINE IN THE CHICK

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(Received for publication, June 20, 1917)

It has previously been established that the chick requires phenylalanine in its diet (1, 2) as do the rat (3) and mouse (4), but for none of these animals is tyrosine an essential dietary constituent (2-6). Moss and Schoenheimer showed (7), by isotope tracer methods, that an appreciable amount of phenylalanine is converted to tyrosine in the rat even when a large amount of tyrosine is present in the diet. Bauer and Berg (4) considered that for the mouse a lack of tyrosine imposed an additional need for phenylalanine. Almquist and Grau (2) found that chicks fed diets containing 1 per cent DL-phenylalanine alone grew poorer than those fed diets which contained, in addition, 2 per cent L-tyrosine. A high level of DL-phenylalanine (3 per cent) obviated the need for tyrosine. Recently, Womack and Rose (8) demonstrated that in the rat tyrosine is capable of stimulating growth only when phenylalanine is furnished in suboptimum amounts. The present report is concerned with the results of feeding various levels of phenylalanine and tyrosine on the growth of chicks, and with determinations of the percentage retention for tissue formation of these components of the diet.

## *Methods*

White Leghorn chicks were reared on a commercial type of diet until they were 2 weeks old, when they were segregated on the basis of weight and were given the experimental diets, which consisted of the following ingredients (in gm): DL-alanine 1.5, L-arginine monohydrochloride 1.2, L-cystine 0.5, L-glutamic acid 5.0, glycine 1.8, L-histidine monohydrochloride monohydrate 0.8, DL-isoleucine 1.0, L-leucine 1.5, L-lysine monohydrochloride 1.4, DL-methionine 0.6, L-proline 2.0, DL-threonine 1.3,<sup>1</sup> DL-tryptophan 0.5, DL-valine 1.5, sodium bicarbonate 1.5, cellulose<sup>2</sup> 5, calcium gluconate 8, mineral mixture 3.8, crude soy bean oil 5, sardine oil (400 vitamin D and 3000 vitamin A units per gm) 0.25, natural mixed tocopherols<sup>2</sup> 0.05, choline chloride<sup>3</sup> 0.2, inositol 0.1, cholic acid 0.1, 2-

<sup>1</sup> Cellu flour

<sup>2</sup> Natural mixed tocopherols (15 per cent), Distillation Products, Inc.

<sup>3</sup> Choline chloride was provided by the Lederle Laboratories Division, American Cyanamid Company, through the courtesy of Dr. T. H. Jukes.

methyl-1,4-naphthohydroquinone diacetate 0 001, thiamine 0 001, riboflavin 0 001, pyridoxine 0 001, nicotinic acid 0 003, calcium (d) pantothenate 0 003, biotin<sup>4</sup> 0 00001, pteroylglutamic acid<sup>5</sup> 0 0001, and glucose<sup>6</sup> to make 100 gm. The mineral mixture contributed the following materials,<sup>7</sup> in gm. to each 100 gm. of diet: tricalcium phosphate 2 0, dipotassium phosphate 0 5, potassium chloride 0 3, manganese 0 01, silicon 0 046, magnesium 0 048, aluminum 0 008, iron 0 014, copper 0 001, zinc 0 001, iodine 0 0008, and cobalt 0 0005. Supplements consisted of DL-phenylalanine or L-tyrosine or mixtures of these two amino acids. Feed and water were supplied *ad libitum*.

In Experiment 1, pairs of carefully selected chicks weighing 69 to 87 gm. were fed diets containing 1 0 per cent phenylalanine plus 2 per cent tyrosine, or 2 per cent phenylalanine, or 2 per cent tyrosine. The chicks were weighed daily for 8 days. In Experiments 2 and 3, each bird was kept in an individual wire-floored compartment of an electrically heated battery brooder in order that accurate records of feed intake might be obtained. Chicks and feed were weighed daily. After a total of 12 days<sup>7</sup> on the diets, the chicks were killed and the carcasses were analyzed for phenylalanine, tyrosine, and nitrogen by methods previously described (9).

### Results

**Growth Rates**—In Experiment 1, the two chicks fed a diet containing 1 0 per cent DL-phenylalanine and 2 0 per cent L-tyrosine gained 3 8 and 4 3 per cent per day,<sup>8</sup> while four chicks fed 2 0 per cent phenylalanine but no tyrosine gained 3 4, 3 9, 3 9, and 6 1 per cent per day, respectively. These two diets were of approximately equal value in promoting growth. The four chicks fed 2 0 per cent tyrosine but no phenylalanine lost weight (growth rate of -2 5 per cent per day), thus these results confirmed the indispensable nature of phenylalanine in the chick (1, 2).

In Experiments 2 and 3, in which various levels of phenylalanine and tyrosine were fed, the growth rates obtained gave an indication of the levels of these amino acids required for optimum growth. In Experiment 2, a high level of tyrosine (2 2 per cent<sup>9</sup>) was kept constant in all diets,

<sup>4</sup> Biotin was furnished by Merck and Company, Inc.

<sup>5</sup> Pteroylglutamic acid was provided by the Lederle Laboratories Division, American Cyanamid Company, through the courtesy of Dr. E. L. R. Stokstad.

<sup>6</sup> Cerelease.

<sup>7</sup> In Experiment 3, one of each pair of chicks was kept for 24 days before it was killed.

<sup>8</sup> Per cent gain per day = (average gain per day × 100)/(average weight during experiment).

<sup>9</sup> This level of tyrosine is equivalent on a mole basis to 2 0 per cent phenylalanine. In both Experiments 2 and 3, the tyrosine levels used were 110 per cent of the phenylalanine levels.

while the phenylalanine level was varied. Fig 1, A shows the growth rates obtained with the several levels of phenylalanine. Best growth was obtained when 0.6 to 0.8 per cent phenylalanine was present, at levels above 1.0 per cent, phenylalanine was found to have a distinctly depressing effect on growth. When tyrosine was kept constant at 1.1 per cent (Experiment 3), the minimum required level remained unchanged, and there was no growth-depressing effect of higher levels of phenylalanine. When phenylalanine was held constant at marginal (0.8 per cent) or submarginal (0.1 per cent) levels, while tyrosine was varied, tyrosine had a definite

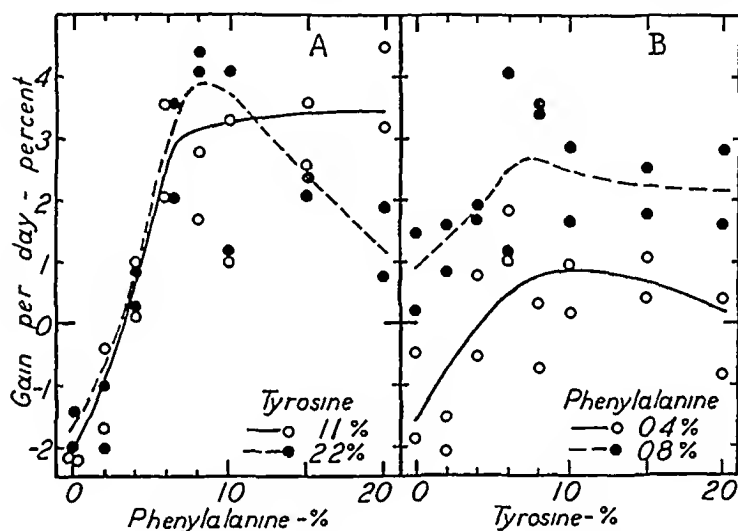


FIG 1, A The effects on individual growth rates of various levels of phenylalanine when the diet contained 2.2 or 1.1 per cent tyrosine

FIG 1, B Individual growth rates of chicks fed various levels of tyrosine when the diet contained 0.4 or 0.8 per cent phenylalanine. The tyrosine (foot-note 9) values are corrected

growth-promoting effect (Fig 1, B). Data are insufficient to show whether there is any difference in the tyrosine requirement between the two phenylalanine levels used.

The data presented in Fig 1 show clearly that the chick has a definite requirement for phenylalanine (0.6 to 0.8 per cent), and that in addition tyrosine or phenylalanine must be supplied at an approximately equal level in order to satisfy the tyrosine requirement. This is analogous to the sulfur amino acid requirements, in which the cystine requirement can be satisfied by cystine or methionine, but only methionine can satisfy the methionine requirement for tissue formation.

*Feed Consumption*—In Experiment 2 feed consumption of all chicks was

low for the 1st day, thereafter there were individual daily fluctuations which are not of importance to this study. In Experiment 3 this lag in consumption was eliminated by feeding a diet of a consistency similar to the amino acid diet before the experiment was started. The actual amounts are important in so far as they allow calculation of the amino acid and nitrogen intakes.

*Retention of Ingested Amino Acids*—From the feed consumption data, it was possible to calculate the amounts of phenylalanine, tyrosine, and nitrogen ingested. From the initial chick weight, the amino acid content of each carcass was estimated (9), and the actual amount was determined by analysis at the end of the experiment.<sup>10</sup> The difference between the initial and final contents (the amount retained) was then divided by the amount ingested and multiplied by 100 to give the per cent retained of nutrient consumed. Some of these percentages were negative, and some were indeterminate (when a phenylalanine-free diet was fed, the percentage of phenylalanine retained could not be calculated).

The retention values for the amino acids are actually *minimum* values for the efficiencies of utilization, for they are the recoveries of the amino acids themselves. Any phenylalanine transformed into tyrosine or other amino acids is not included in the percentage retained, yet this amount would have to be included if a value were to be called an efficiency of utilization.<sup>11</sup>

In Fig. 2 the results of these retention calculations are presented for Experiment 2, in which 2 per cent tyrosine (corrected) was a constant component of the diets. The curve for phenylalanine rose rapidly from a large negative value to about 35 per cent at 0.6 per cent phenylalanine, after which it decreased to about 7 per cent at 2 per cent phenylalanine. The nitrogen retention followed a similar course, but did not decrease as much as did phenylalanine at the higher levels. This level of tyrosine did not allow its efficient use at any level of phenylalanine, the highest value observed was 9 per cent.

When a lower level of tyrosine was employed (Experiment 3, Fig. 3),

<sup>10</sup> The contents of the carcasses at the end of the experiment were determined by analysis rather than by calculation from body weight and previously published data (9), because the effect of the diet on the composition of the body proteins was not known (cf. also "Constancy of tissue protein composition," below).

<sup>11</sup> The work of Moss and Schoenheimer (7) indicated that more than 25 per cent of ingested phenylalanine is transformed to tyrosine and incorporated into the body proteins of growing rats fed a diet adequate in both these amino acids. A calculation of the chick results on the basis of this work increases the phenylalanine utilization from the retention value of 14 to 21 per cent, and decreases the tyrosine retention from 18 to 5 per cent. These results are calculated from the chicks of Experiment 3 which were fed 1.1 per cent tyrosine and 2.0 per cent phenylalanine.

the same curve forms were found. The phenylalanine retention decreased as the diet became overloaded with this amino acid, but the nitrogen retention did not decline. It will be recalled that rate of growth was adversely affected by high phenylalanine levels when tyrosine was present at the 2 per cent level, but not at the 1 per cent tyrosine level.

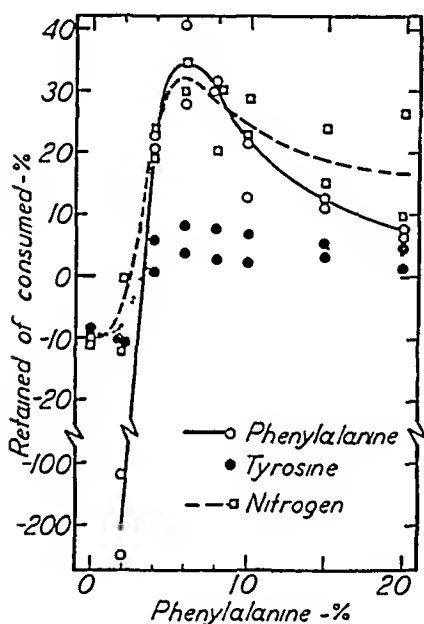


FIG 2

FIG 2 The effects of the phenylalanine content of the diet on the percentage nutrient retained of that consumed when all diets contained 2.2 per cent tyrosine.

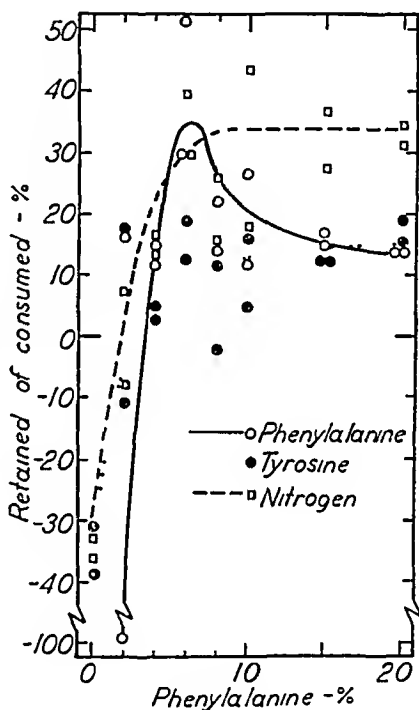


FIG 3

FIG 3 The effects of the phenylalanine content of the diet on the percentage nutrient retained of that consumed when all diets contained 1.1 per cent tyrosine.

As would be expected, the tyrosine retention was higher than before, but still below the nitrogen retention.

In both experiments (Figs 2 and 3) the optimum percentage retention of phenylalanine was practically identical with that of nitrogen. If only one of the phenylalanine isomers were being used for tissue formation, the phenylalanine retention would be only about half the nitrogen retention. Thus it appears that the isomers of phenylalanine are of equal value in promoting growth in the chick, as they are in the rat (10) and mouse (4).

These retentions were approximately equal to those obtained when the diets fed were of a purified type, or composed of natural feedstuffs<sup>12</sup>

When the phenylalanine level was suboptimum (0.4 per cent, Fig 4), nitrogen retention was markedly increased as the dietary tyrosine level was increased, although this retention curve was definitely below that obtained with 0.8 per cent phenylalanine. The phenylalanine curves of

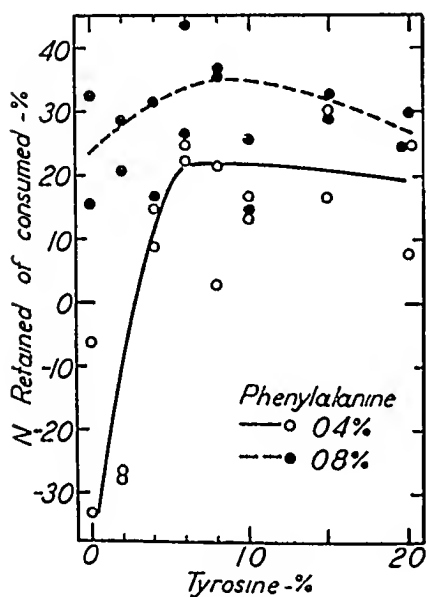


FIG 4

FIG 4 The effects of various levels of tyrosine (foot-note 9) on the percentage retention of nitrogen when the phenylalanine content of the diet was marginal (0.8 per cent) or submarginal (0.4 per cent)

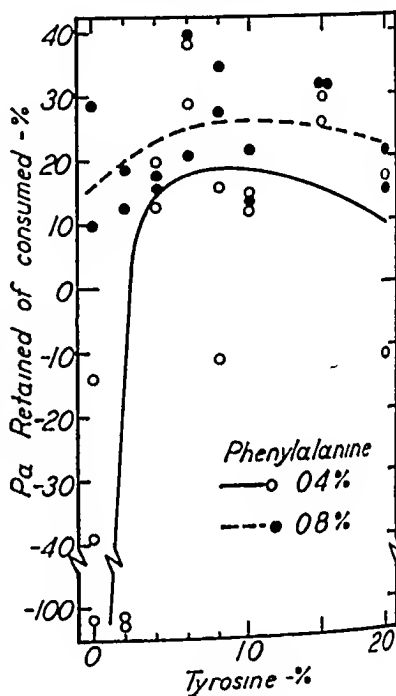


FIG 5

FIG 5 The relation of the tyrosine content of the diet to the percentage retention of phenylalanine (Pa) at two phenylalanine levels

Fig 5 show that tyrosine aided in the retention of phenylalanine at both levels of phenylalanine, but that a greater effect was produced when the phenylalanine level of the diet was suboptimum

<sup>12</sup> A purified diet, in which all the 20 per cent protein was provided by a mixture of casein, arginine, cystine, and glycine, yielded the following retentions (the average and the range of four chicks) nitrogen 40 per cent (34 to 43), phenylalanine 32 per cent (27 to 35), tyrosine 20 per cent (18 to 22). Casein contributes slightly more phenylalanine to the diet than is necessary to satisfy the chick requirements, hence the phenylalanine retention is lower than that of nitrogen

It was indicated in Figs 2 and 3 that dietary tyrosine was never efficiently retained when high levels of tyrosine were present in all diets. When only a minimum level of phenylalanine was fed, an appreciable amount of ingested tyrosine was retained at low tyrosine levels (Fig 6). The most efficient retention of tyrosine was found when the diet contained marginal phenylalanine and insufficient tyrosine for best growth.

*Constancy of Tissue Protein Composition*—The extent to which an animal existing under the stress of a deficient diet can change the amino acid contents of its tissue protein is not well known. Considerable work has been done on this subject, involving principally carcass analyses for ni-

TABLE I

*Relation of Diet to Amino Acid Composition of Carcass Crude Protein (N  $\times$  6.25)*

The deficient diets contained 0 to 0.4 per cent of the amino acid, optimum 0.6 to 1.0 per cent, excess 1.5 to 2.0 per cent

Phenylalanine	Tyrosine	Amino acids in carcass protein	
		Phenylalanine	Tyrosine
		per cent	per cent
Deficient	Deficient	4.9	2.9
"	Optimum	4.9	3.0
"	Excess	4.8	3.0
Optimum	Deficient	4.9	2.8
"	Optimum	4.7	2.8
"	Excess	4.6	2.9
Deficient	Optimum	5.0	2.8
Optimum	"	4.9	2.8
Excess	"	5.2	3.0

trogen and sulfur rather than for individual amino acids. The early work (reviewed by Cathcart (11) and Lee and Lewis (12)) yielded no unequivocal answer. Consequently Lee and Lewis (12) investigated the problem by analyzing carcasses of rats that were fasted, or fasted and refed, or fed a cystine-deficient diet. The amino acids studied were cystine, tyrosine, and tryptophan, and in these no deviations from normal were found under any of these conditions.

Pertinent data obtained from Experiment 3 are presented in Table I, from which it is apparent that there is no consistent effect of the various dietary levels on the levels found in the protein. There are several conclusions to be drawn from this fact: (1) The animal must fit the amino acids available into a rigid pattern of amino acids in tissue protein, if the diet does not contain a particular amino acid, either the animal must manufacture it, or new tissue will not be formed, and old tissue will break

down (2) There is no appreciable amino acid storage This has long been apparent from the rapid effect on growth noted after an animal is placed on an amino acid-deficient diet (3) When fed a diet deficient in an essential amino acid, the animal wastes all amino acids, for the proportions are kept constant in its tissues (4) Finally, and perhaps most important, we are justified in considering the rate of growth as the most important criterion of dietary protein adequacy, because it is a measure of the actual amount of amino acids laid down in the tissue proteins

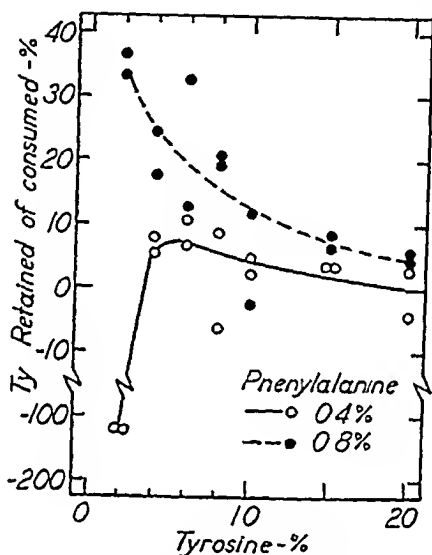


FIG 6 The relation of the tyrosine content of the diet to the percentage retention of tyrosine (Ty) at two phenylalanine levels

#### SUMMARY

The chick requires 0.6 to 0.8 per cent DL-phenylalanine in its diet for optimum growth, plus an equal amount of either phenylalanine or L-tyrosine to provide for the tyrosine requirement. Tyrosine can thus spare phenylalanine only when marginal or submarginal levels of phenylalanine are present.

Under optimum conditions, approximately 35 per cent of the ingested phenylalanine is incorporated into the body proteins. Because this percentage is practically identical with the per cent of nitrogen retained, it is concluded that both optical isomers of phenylalanine are used by the chick, as they are in the rat and mouse. Tyrosine is not efficiently retained unless the phenylalanine level for growth is marginal and the tyrosine level is submarginal.

The phenylalanine and tyrosine contents of the diets had no effect on the levels of these amino acids in the tissue proteins.



This investigation was aided by a grant from the Nutrition Foundation, Inc

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# A COLORIMETRIC PROCEDURE FOR THE DETERMINATION OF SMALL AMOUNTS OF FATTY ACID

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(Received for publication, July 14, 1947)

The basis of the method is the measurement of the color change produced by the reducing action of fatty acid or cholesterol on a sulfuric acid-dichromate mixture. This reaction has been used in several ways for the determination of fatty acids and other organic material. Bang (1) made it the basis of a micromethod by titrating the excess of dichromate with starch-iodine and thiosulfate. In this laboratory (2) the procedure of Bang was modified to make the oxidation complete. Van Slyke and Folch (3) used the reagent by collecting and measuring the carbon dioxide evolved in the oxidation. As far as can be determined, no one has reported the use of the color change in the reagent as a means of measurement of fatty acids or other organic material, and, since it was found that the change was closely proportional to the amount of fatty acid oxidized and that the actual determination was rapid and easy to carry out, a procedure was developed and reported (4). Since the reagent is sensitive to organic matter of all kinds, it is necessary to isolate the fatty acids before measurement. The procedure for the isolation has been worked out by previous investigators and need not be reported here. The change in color cannot be measured in the Duboseq or similar visual colorimeters, but it can be determined by the photoelectric type of instrument. For greater sensitivity a depth of liquid greater than in the ordinary photoelectric colorimeter was desirable and a special colorimeter was devised and is described below.

**Reagent**—The Nicloux type of sulfuric acid-dichromate reagent was used. It is made by dissolving 10 gm. of  $\text{AgNO}_3$  and 10 gm. of  $\text{K}_2\text{Cr}_2\text{O}_7$  separately in water, mixing the solutions, and purifying the precipitated silver dichromate by centrifuging and washing the precipitate two or three times with water in the centrifuge. 100 cc. centrifuge tubes were found convenient for mixing and washing. The separated precipitate was dissolved in 500 cc. of concentrated  $\text{H}_2\text{SO}_4$  and kept in a glass-stoppered bottle.

**Solvent**—Either purified petroleum ether or a mixture of 7 parts of petroleum ether to 1 of chloroform can be used. The latter is more satisfactory, since it does not easily form emulsions in extracting lipide from either acid or alkaline solutions.

### Procedure

The material to be determined (fatty acid or cholesterol), dissolved in petroleum ether or petroleum ether-chloroform (7 1), was made to a volume such that 1 cc contained about 0.1 mg of the substance to be measured. From this solution an aliquot containing 0.4 to 0.5 mg was measured into a 50 cc beaker and the same volume of solvent alone was measured into another beaker. The solvents were evaporated by immersion in boiling water, after which 3 cc of the reagent were measured into the beakers and the mixture digested for 15 minutes in a closed vessel set in boiling water. At the end of the period 3 cc of distilled water were added and the mixture set aside for 10 minutes to cool, after which it was transferred to the colorimeter cups and readings made. The values were determined by the use of a calibration curve. Care was taken during the evaporation of the solvent not to expose the tiny residues to heat and air longer than necessary to remove the solvent completely, since even the higher fatty acids are appreciably volatile and oxidizable under these conditions. Slight changes occur in the diluted mixture on standing, so that it is advisable to make the readings near the 10 minute point. Measurement of the dichromate reduced and hence of the amount of lipid present can be made by titration with thiosulfate and starch in the usual way. By this means it was found that, by the procedure given, the fatty acid or cholesterol was oxidized to the extent of about 88 per cent of the theoretical value, and the amount of reagent used constituted an excess of about 4 times that needed for this oxidation.

*Calibration Curves for Fatty Acid or Cholesterol*—For this purpose fatty acid or cholesterol was dissolved in petroleum ether or petroleum ether-chloroform (7 1) and made to volume such that 1 cc of the solution contained 0.1 mg of the standard substance.

Either palmitic, stearic, or oleic acid may be used as standard, since the differences in values are within the limit of error of the method. As cholesterol does give significantly higher values, a separate curve was run for it. For the calibration, from 1 to 8 cc of the standard solutions was measured into one of the 50 cc beakers and the same amount of solvent into another. After evaporation of the solvent the residues were treated as directed in the procedure and readings were made. Eight or ten determinations were made at each level and the positions marked on coordinate paper. The results are shown on the calibration chart. The accuracy of the measurements was about  $\pm 5$  per cent. The curves obtained are shown in Fig 1.

*Special Colorimeter*—In most of the photoelectric colorimeters at present available the intensity of color is measured by passing the light across small tubes (about 25 mm in diameter). The sensitivity may be increased by the use of a greater thickness of solution, such as is obtained in the

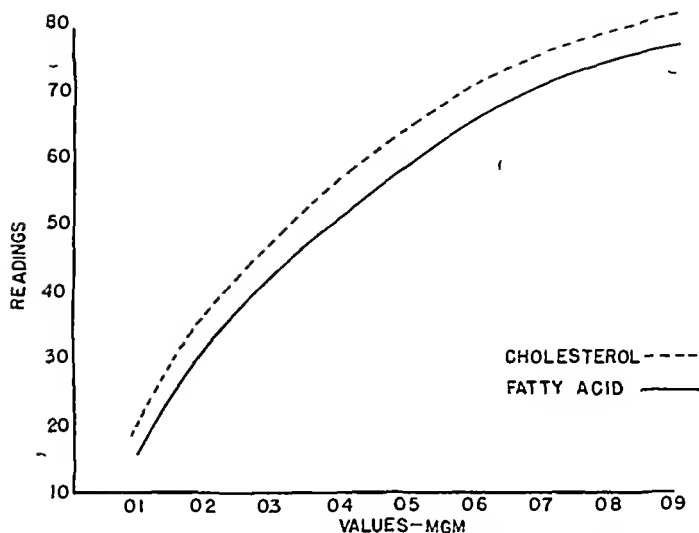


FIG 1 Calibration curves for cholesterol and fatty acid

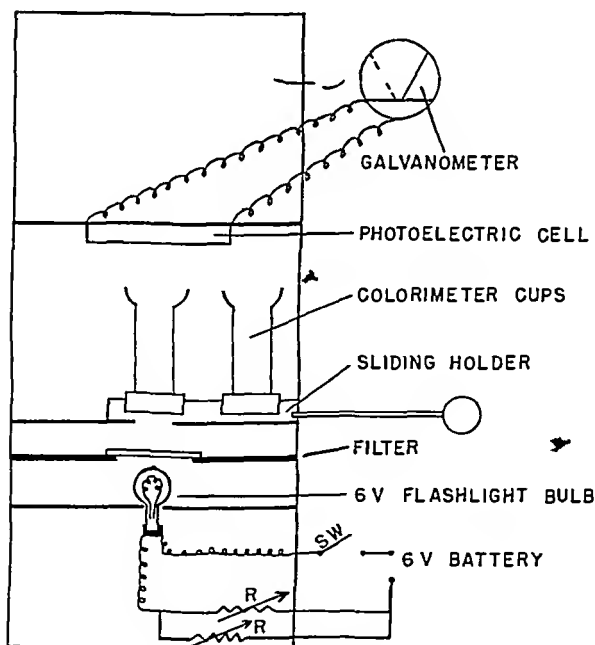


FIG 2 Diagram of the special colorimeter

cups used in the visual colorimeters. Such cups are used in the instrument described below. No claim can be made for originality in idea and very

little in device for this instrument, since a similar one has already been described (5), but the simplicity of construction, ease of use, and versatility of the present instrument are believed to warrant its description

A diagram of the special instrument is shown in Fig 2. It consists of an oblong box about  $4\frac{1}{2} \times 5 \times 7$  inches with a light-tight door. The working parts (holders) are made of  $\frac{3}{32}$  inch bakelite or similar material, sliding in and out of grooves in the inside of the box. The light system is a modification of that described by Evelyn (6) and consists of a 6 volt flashlight bulb with a 6 volt automobile battery as the source of current. In the present work it was necessary to keep the battery fully charged, so that a permanent connection with a charger is desirable. The colorimeter cups are carried in a slide mounted on another holder which has an opening directly over the light source. The slide is operated from outside the box and is so constructed that first the cup containing the blank and after adjustment the cup containing the solution to be measured are centered over the light. A light filter and the flashlight bulb are mounted in similar holders beneath the cups, as shown in Fig 2. (In the present work no filter was found which gave better results than the unfiltered white light.)

Situated a short distance above the colorimeter cups is the photoelectric cell (Weston model 594, type 2, 668-0) connected to a galvanometer of the ordinary student type (Leeds and Northrup type P).

In operation the colorimeter cups containing the blank and the solution to be measured are mounted in the sliding plate and the cup containing the blank solution is pushed over the light. The galvanometer is adjusted by means of the variable resistances  $R$  and  $R_1$  to a convenient point on the scale and let stand for a minute or two to come to rest, then after final adjustment the cup containing the solution to be tested is pushed into place and readings made. The difference between this reading and the reading set for the blank is the reading for the solution being tested. The values are then read off the calibration curve.

#### SUMMARY

A method is described for the colorimetric determination of small amounts of fatty acid or cholesterol, making use of a special type of photoelectric colorimeter.

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# THE CLEAVAGE OF PHOSPHOLIPIDES BY BRAIN TISSUE\*

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(Received for publication, July 16, 1947)

Studies of brain metabolism with isotopic indicators are unanimous in showing that the rate of turnover of lipides in the adult rat brain is exceedingly slow *in vivo* (1-6). The results obtained by several investigators with  $P^{32}$  are particularly convincing in demonstrating a low rate of phospholipide metabolism. It might be expected from these findings that no change in lipides, detectable with conventional methods, would occur during short periods of incubation of brain preparations *in vitro*. However, the chemical behavior of isolated tissue is not necessarily the same as it is in its natural environment in the living animal, and it seemed worth while to include some measurements of lipides in a study of brain respiration under way in our laboratory.

These preliminary experiments showed an apparent decrease in the phosphorus of the fraction of brain homogenates, soluble in organic solvents, during a 4 hour period of incubation. This finding was confirmed and studied in considerable detail.

## EXPERIMENTAL

Brain tissue from rats was homogenized with the all-glass apparatus of Potter and Elvehjem (7) in saline-carbonate buffer, saturated with 5 per cent  $\text{CO}_2$ -95 per cent  $\text{O}_2$ . In the experiments through Experiment 45 (Table I) the buffer was at room temperature and the homogenizer was run from 40 to 50 seconds. The tube was moved up and down continuously during the homogenization. The temperature did not increase perceptibly to the touch. However, to minimize possible effects of frictional heating, from Experiment 45 through the rest of the investigation cold buffers were used and the time of homogenization was reduced to 25 to 30 seconds. For control determinations portions of the homogenates, as shown in Table I, were pipetted into 25 cc volumetric flasks and extracted at once with alcohol-ether (3:1). In Experiments 10 to 14, inclusive, other portions were employed for respiratory measurements in the Warburg apparatus,

\* Preliminary reports of this investigation were presented to the Division of Biological Chemistry of the American Chemical Society at Buffalo, 1942, and to the American Society of Biological Chemists (*Federation Proc.*, 2, 70 (1943)).

TABLE I

*Decrease in Lipid Phosphorus during Incubation of Brain Tissue Homogenates in Carbonate Buffer for 4 Hours with Shaking at 37.5°*

Experiment No	Tissue	Weight of brain† per cc homogenate	Volume of homogenate†	Phosphorus per mg brain		Decrease per cent
				Control	Incubated	
		mg	cc	γ	γ	
10	Whole brain	131	1.0	2.64	2.40	9.1
11	" "	130	1.0	2.57	2.33	9.3
12	" "	131	1.0	2.61	2.52	3.4
13	" "	131	1.0	2.64	2.24	15.2
14	" "	48	2.0	2.53	2.29	9.5
15	Cerebral hemispheres	133	1.0	2.53	2.15	15.0
16	" "	131	1.0	2.48	2.31	6.9
17	" "	131	1.0	2.48	2.27	8.5
27	" "	83	2.0	2.61	2.41	7.7
29	" "	91	2.0	2.57	2.49	3.1
30	" "	90	2.0	2.25	2.15	4.4
31	" "	158	0.5	2.37	1.96	17.3
32	" "	173	0.5	2.33	2.16	7.3
33§	" "	168	0.5	1.61	1.35	16.1
34§	" "	189	0.5	1.76	1.56	11.4
35§	" "	199	0.5	1.76	1.67	5.1
36	Cerebellum + brain stem*	153	0.5	2.51	2.28	9.2
37	Cerebral hemispheres	148	0.5	2.27	2.18	4.0
38	" "	168	0.5	2.30	1.99	13.5
39	" "	153	0.5	2.60	2.25	13.5
40	" "	167	0.5	2.37	2.29	3.4
41	" "	167	0.5	2.27	2.18	4.0
41	Cerebellum + brain stem*	166	0.5	2.55	2.45	3.9
42§	Whole brain	167	2.0	1.62	1.53	5.6
43	" "	141	0.5	2.50	2.39	4.4
45	" "	177	0.5	2.52	2.30	8.7
46	" "	164	0.5	2.35	2.24	4.7
47	" "	147	0.5	2.49	2.29	8.0
53	" "	165	0.5	2.44	2.16	11.5
55	Cerebral hemispheres	162	0.5	2.30	2.13	7.4
55	Cerebellum + brain stem*	137	0.5	2.69	2.55	5.2
56	Whole brain	172	0.5	2.39	2.17	9.2

\* Including medulla and pons

† The data in this column are only approximate, but errors in this measurement have no effect on the percentage decrease, since they apply equally to both of the phosphorus values

‡ The volumes shown were pipetted into 25 cc volumetric flasks for control determinations and for incubation, except in Experiments 10 to 14, in which the incubated samples were pipetted at the end of the incubation period

§ Age of rats: Experiment 33, 18 days; Experiments 34 and 35, 20 days; Experiment 42, 16 days; other rats adults



and at the end of a 4 hour period of shaking in the bath at 37.5° samples were pipetted for extraction exactly as in the control analyses. In the remaining experiments "control" and "incubated" portions were pipetted into 25 cc volumetric flasks at the start of the experiment. The "control" samples were extracted at once, the experimental samples were shaken for 4 hours in the Warburg apparatus and then extracted. The stoppers were removed and the flasks were flushed out with CO<sub>2</sub>-O<sub>2</sub> at intervals of about ½ hour during the period of incubation.

In making the extractions about 15 cc of solvent were added and brought to a boil and the flasks were allowed to stand, usually overnight. Solvent was added to the mark and the contents were thoroughly mixed and filtered. Phosphorus was determined in 1 cc portions of the filtrate by Sperry's micro modification (8) of the Fiske and Subbarow method (9).

In most of the experiments two control and two incubated samples were extracted and each sample was analyzed in duplicate, *i.e.*, each phosphorus value, with a few exceptions, is the average of four determinations. An estimate of the error of the procedure for determining phosphorus in the extracts was obtained by calculating the standard deviation of the percentage deviations of the duplicate estimations from their respective means. The standard deviation of 344 such values was  $\pm 1.1$  per cent. This estimate does not include the errors of preparing the extracts, particularly of pipetting samples of the homogenates which usually contained air bubbles and were not entirely homogeneous<sup>1</sup>. An estimate of the over-all error was obtained by calculating the standard deviation of the four analyses (two on each of two samples) from their respective means. The value was  $\pm 1.9$  per cent.

Although in some of the experiments the decreases in lipid phosphorus content (Table I) were not significant, the majority were too large to be fortuitous and the fact that a change in the same direction was observed in every experiment leaves no doubt that the over-all result is significant. The wide variation in the magnitude of the change is probably related to variations in the preparation of the homogenates.

The loss of lipid phosphorus during incubation of brain tissue appears to demonstrate the presence in brain of a fairly active mechanism capable of cleaving phospholipides, but other explanations were considered. That non-lipid phosphorus was extracted in the control but not in the incubated samples appeared unlikely from some experiments in which the filtered alcohol-ether extracts from replicate control and incubated samples were taken to dryness and reextracted with ethylene chloride. The solutions were made up to the original volume and phosphorus was determined in

<sup>1</sup> In the latter part of the work, starting with Experiment 71, the samples of the emulsions were weighed to minimize this error.

1 cc portions. A comparison of the results (Table II) with the corresponding values found in alcohol-ether extracts of the same homogenates (Table I) shows that although only about three-fourths of the alcohol-ether soluble phosphorus was extracted by ethylene chloride the percentage differences between control and incubated homogenates were as large as, or in some experiments considerably larger than, those found in the alcohol-ether extracts.

More serious is the possibility that a portion of the phospholipides became insoluble during incubation through the formation of stable combinations with protein, or in some other way. Three types of evidence, bearing on this point, were obtained. In one series of experiments a different and somewhat more efficient solvent mixture, methyl alcohol-chloroform (1 1),

TABLE II  
*Reextraction with Ethylene Chloride*

Experiment No	Phosphorus per mg. brain		Decrease	Experiment No	Phosphorus per mg. brain		Decrease
	Control	Incubated			Control	Incubated	
	$\gamma$	$\gamma$	per cent		$\gamma$	$\gamma$	per cent
32	1.62	1.49	8.0	35	1.48	1.30	12.2
33	1.33	1.13	15.0	39S*	1.85	1.64	11.4
34	1.34	1.06	20.9	42	1.30	1.18	9.2

\* In this experiment rat blood serum was added to the homogenate. In the corresponding experiment, in which alcohol-ether was used as the only solvent, the decrease was 10.0 per cent.

was compared with alcohol-ether. The technique was the same as that described except that incubation was continued for 21 to 24 hours, with or without shaking during the first 6 or 7 hours, and the more complex carbonate buffer of Krebs and Henseleit (10), with phosphate omitted, was used in Experiments 79 to 88. Merthiolate was added in the proportion of 10 mg to 100 cc of buffer. Each phosphorus value (Table III) is the average of at least four determinations, two on each of two samples of the brain homogenate. Although methyl alcohol-chloroform extracted a little more phosphorus than alcohol-ether, the decreases were about the same with the two solvent mixtures, except in Experiment 87 in which a very high apparent decrease was found in the samples extracted with alcohol-ether. This value is so far out of line with other results obtained in this investigation that it is open to serious question, but the replicate determinations agreed closely and there is no evident reason for excluding it.

In a further effort to rule out a change in solubility as an explanation of the results an entirely different method of extraction was employed, *viz*, the

colloidal iron procedure of Folch and Van Slyke (11) In the first attempts to apply this technique to brain homogenates the agreement among replicates was poor and the amounts of lipid phosphorus found were only about half of those obtained with direct alcohol-ether extraction The method was studied in considerable detail, with particular attention to the procedure for extraction from the colloidal iron precipitate Various amounts of water were added to the precipitate and various combinations of solvents

TABLE III  
*Comparison of Solvents*

Experiment No	Alcohol ether (3:1)			Methyl alcohol chloroform (1:1)		
	Phosphorus per mg brain		Decrease	Phosphorus per mg brain		Decrease
	Control	Incubated		Control	Incubated	
	$\gamma$	$\gamma$	<i>per cent</i>	$\gamma$	$\gamma$	<i>per cent</i>
51	2 32	1 97	15 1	2 44	2 12	13 1
51B*	2 46	1 88	23 6	2 65	2 19	17 4
52	2 35	2 03†	13 6	2 55	2 20	13 7
52B*	2 31	1 74	24 7	2 66	2 05	22 9
79‡	0 98	0 82	16 3	1 06	0 88	17 0
81	2 42	2 08	14 0	2 53	2 18	13 8
82	2 09	1 97	5 7	2 38	2 18	8 4
87	2 33	0 90	61 4§	2 78	2 49	10 4
88	2 16	1 87	13 4	2 28	1 97	13 6

\* Rat blood was added to the brain homogenates in these experiments

† The phosphorus value found in one of the samples was so low (1 56  $\gamma$ ) that an error seemed likely and the value was omitted from the average

‡ The brains of 5 day-old rats were used

§ The individual phosphorus values were as follows: control Flask 1, 2 32 and 2 33, control Flask 2, 2 34, and 2 34, incubated Flask 1, 0 91 and 0 89, incubated Flask 2, 0 89 and 0 90

were tried Fairly uniform results were obtained with the following procedure Approximately 0 2 cc portions of the homogenate were placed in tared 15 cc centrifuge tubes, weighed, and treated as described by Folch and Van Slyke The precipitate was washed twice with magnesium sulfate solution and once with water Either 0 8 or 1 1 cc of water and 2 cc of alcohol were added and the precipitate was stirred into suspension and transferred to a 10 cc volumetric flask Two 1 cc portions of alcohol followed by 2 and 1 cc portions of ether were used in washing The flask was filled to the mark with ether and 1 0 cc portions of the filtrate were taken for phosphorus determination In the preliminary experiments the quantity of water added was varied from none to 1 6 cc Maximal yields of lipid phosphorus were obtained with 0 8 and 1 1 cc and both of these

quantities were used in the incubation experiments. In most instances the recovery was around 65 to 70 per cent of the directly extractable phosphorus, though occasionally values up to approximately 80 per cent were found. Despite this large discrepancy the percentage decrease during incu-

TABLE IV  
*Extraction with Folch-Van Slyke Procedure*

Experiment No	Extraction procedure	Water added	Phosphorus per mg brain		Decrease  per cent
			Control	Incubated	
		cc	$\gamma$	$\gamma$	
S1	Direct		2.42	2.08	14.0
	Folch-Van Slyke	0.8	1.59	1.40	11.9
	"	1.1	1.72	1.41	16.3
S2	Direct		2.09	1.97	5.7
	Folch-Van Slyke	0.8	1.43	1.35	5.6
	"	1.1	1.55	1.57	0
S7	Direct		2.33	0.90	61.4
	Folch-Van Slyke	1.1	1.91	0.71	62.8
S8	Direct		2.16	1.87	13.4
	Folch-Van Slyke	0.8	1.31	1.25	6.7
	"	1.1	1.41	1.40	0

TABLE V  
*Behavior of Alcohol-Ether-Soluble Lipides of Brain Tissue Homogenates during Incubation for 4 Hours*

Experiment No	Lipides per mg brain		Change	Experiment No	Lipides per mg brain		Change
	Control	Incubated			Control	Incubated	
	$\gamma$	$\gamma$	per cent		$\gamma$	$\gamma$	per cent
10	180	179	-0.6	17	160	165	+3.1
12	175	184	+5.1	43	190	187	-1.6
13	160	160	0	45	170	172	+1.2
15	157	146	-7.0	46	168	164	-2.4
16	157	151	-3.8	47	175	179	+2.3

bation of 0.2 cc portions of the homogenates in centrifuge tubes was usually of the same order of magnitude as that found in replicate samples of the same homogenate extracted directly (Table IV). The finding of remarkably high values with both procedures in Experiment S7 is particularly striking (cf Table III).

A third type of evidence against the interpretation of the result on the basis of a change in solubility was obtained from measurements of the total alcohol-ether-soluble lipides in some of the experiments (Table V). The

total extract remaining after the two 1 cc samples for phosphorus determination were removed was transferred quantitatively to a weighed flask and taken to dryness in a stream of carbon dioxide. Although the quantities were small, close agreement between duplicate determinations was obtained with the use of a uniform technique for drying and weighing the flasks. The apparent changes were about equal in both directions and were within the error of the procedure, the largest apparent difference (7 per cent) represented a weight of only 1.4 mg.

TABLE VI

*Effect of Blood Added to Homogenates of Brain Tissue on Cleavage of Phospholipides*

Experiment No	Time of incubation	Blood per gm brain	Decrease in phosphorus	Experiment No	Time of incubation	Blood per gm brain	Decrease in phosphorus
	hrs	cc	per cent		hrs	cc	per cent
37	4	0	4.0	53	4	0	11.5
	4	0.85	6.5		4	0.22	4.9
38	4	0	13.5		24	0	16.0
	4	0.28	7.6		24	0.22	20.9
39	4	0	13.5	54	7	0	9.3
	4	0.41*	10.0		7	0.23	7.0
48	24	0	25.1		24	0	14.8
	24	0.24	24.1		24	0.23	24.2
49	24	0	7.5	56	4	0	9.2
	24	0.25	17.4		4	0.24	6.3
51	24	0	15.1		21	0	9.4
	24	0.24	23.6		21	0.24	13.6
52	24	0	13.6				
	24	0.29	24.7				

\* Blood serum from another rat added in this experiment

*Effect of Added Blood*—The possibility that phospholipide-splitting enzymes of the blood were responsible for the decrease in lipid phosphorus was investigated through a study of the effect of a large excess of blood. The brain was divided longitudinally into approximately equal parts, of which one was homogenized in carbonate buffer, and the other in buffer to which had been added blood from the same rat, or in one case serum from another rat. Samples of the two homogenates were carried through the procedure side by side. The flasks were shaken in the Waring bath for the times indicated in Table VI, except that in the 21 or 24 hour experiments shaking was continued for 4 to 7 hours, after which the flasks were placed in an incubator at 37.5° for the remainder of the period. The data show no effect of blood or serum in increasing the cleavage of phospholipides during a 4 or 7 hour period of incubation, indeed in five of the six experiments the decrease in phosphorus was less in the presence of an excess of

blood than in the emulsions to which no blood had been added. This result indicates that enzymes of blood were not responsible for the cleavage of phospholipides found in short time experiments. On the other hand in six of the seven 21 or 24 hour experiments the decrease was greater in the presence than in the absence of added blood, a finding which suggests that enzymes of the blood may be responsible, at least in part, for the increased splitting of phospholipides usually observed during autolysis for a long period.

*Effect of Time of Incubation and of Shaking*—In several experiments the effect of time of incubation (1 to 24 hours), of shaking, or of both was studied on replicate samples of the same homogenate. The data are too variable to justify detailed presentation, but they show a definite tendency for the degree of cleavage to increase with increasing time and little, if any, effect of shaking.

*Effect of Heating*—Replicate samples of the homogenates were heated at 100° for 2 to 10 minutes before incubation in several of the experiments. In all cases the splitting of phospholipides was reduced, usually to less than half that of the unheated samples, but in only one instance was there complete inactivation. Some of this cleavage probably occurred during the heating, in four experiments in which samples were heated to 100° for 10 minutes and then analyzed without incubation, there was a decrease in phosphorus averaging 3.2 per cent. However, not all of the splitting in the heated and incubated samples can be accounted for in this way, and it must be concluded that if the reaction is catalyzed by enzymes they must be unusually resistant to heat. There is some support for this possibility: the lecithinase A of snake venom and of pancreas is remarkably stable to heat (12, 13).

*Effect of Various Solutions Used in Preparing Brain Homogenates*—In the experiments described above either a simple saline-carbonate buffer or the more complex carbonate buffer described by Krebs and Henseleit (10), with phosphate omitted, was used. These buffers were saturated with 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub>. They were compared in eight experiments in which half the brain was homogenized in one and the other half in the other buffer. The two homogenates were incubated side by side for different periods of time, with or without shaking. The average percentage decreases in phosphorus were 12.1 and 13.8, respectively. In several of these experiments the pH was determined with the glass electrode in replicate samples which had been carried through the incubation procedure. The values ranged from 7.0 to 7.9 and agreed closely for the two homogenates in each experiment.

In three experiments homogenates made from halves of the same brain in simple carbonate buffer and in water were compared. The average de-

crease in phosphorus in the carbonate homogenates was 9.7 per cent, while in the water homogenates no change occurred. The pH values in the incubated carbonate homogenates were 7.7, 7.4, and 7.4, in the water homogenates the values were 6.8, 7.0, and 7.2, respectively. Although the pH was lower in the water than in the carbonate homogenates, the absence of phospholipid cleavage in the former cannot be attributed to the small difference in acidity, since no relationship between degree of splitting and pH within this range was evident in the experiments described in the preceding paragraph. The negative result with homogenates prepared in water suggests

TABLE VII  
*Incubation of Minced Brain Suspended in Buffer*

Experiment No	Buffer*	Treatment	Decrease in lipid P per cent	Experiment No	Buffer*	Treatment	Decrease in lipid P per cent
71	Saline-carbonate	Shaken	13.9	73	Saline-carbonate	Shaken	1.7
	"	Not shaken	15.5		"	Not shaken	22.7
	Krebs-Henseleit	Shaken	8.0		Krebs-Henseleit	Shaken	6.3
	"	Not shaken	11.6		"	Not shaken	18.8
72	Saline carbonate	Shaken	16.0	74	Saline carbonate	Shaken	7.3
	"	Not shaken	7.1		"	Not shaken	6.3
	Krebs Henseleit	Shaken	0		Krebs Henseleit	Shaken	8.7
	"	Not shaken	0		"	Not shaken	14.5

\* Phosphate was omitted from the Krebs-Henseleit buffer (10)

that the reaction is more complex than a simple hydrolysis and may involve oxidative processes which require intact cells, and it also provides further evidence against the possibility that cleavage of non-lipide esters, such as adenosine triphosphate, was a factor in the decrease of lipid-soluble phosphorus.

In three experiments a veronal buffer (pH 7.4) was used. In one a 9 per cent decrease in phosphorus was found, in the others there was no change. In several experiments phosphate buffers were employed in conjunction with the Folch-Van Slyke extraction procedure (11). No decrease in lipid phosphorus was found except in one experiment in which methyl alcohol-chloroform (1:1) was used for extraction, and this could not be confirmed.

Glucose and nicotinamide, added in some experiments, had no apparent effect on the splitting of phospholipides.

*Decrease in Lipide Phosphorus during Incubation of Minced Brain*—A rat brain was minced with scissors and ground to a fairly homogeneous paste in a mortar under conditions which prevent loss of tissue water (14). Fr

teen portions were drawn up in a glass tube fitted with a glass plunger, transferred to tared 25 cc volumetric flasks, and weighed. Simple saline carbonate buffer was added to half the flasks and the Krebs-Henseleit carbonate buffer to the other half. Of each set of seven, three were extracted at once with alcohol-ether as controls and the other four were incubated for about 21 hours, with or without shaking for the first 5 to 6 hours. Each sample was analyzed for phosphorus in duplicate.

The results (Table VII) show that with three exceptions the lipid phosphorus decreased as it did in similar experiments with brain homogenates. There was no consistent difference in results with the two buffers. Shaking usually, though not always, appeared to have an adverse effect on the reaction.

#### DISCUSSION

The results of this investigation demonstrate that a significant decrease, which may be of considerable magnitude, in the quantity of phosphorus extractable with lipid solvents occurs during incubation of homogenized or minced brain tissue in carbonate buffer, and the findings show further that the decrease is in all probability the result of an active mechanism present in brain tissue with the ability to split phospholipides at a relatively rapid rate.

Other investigators have presented indirect evidence for the presence of "lecithinase" in the brain. Cornat (15) found that choline was liberated during autolysis of a sample of minced brain for 72 hours in the presence of chloroform, and Simon (16) in similar experiments observed an increase in inorganic phosphorus. King (17) incubated aqueous extracts of rabbit and chicken brains with a lecithin emulsion for 48 hours and observed a small and variable increase in the total free and acid-soluble phosphorus. The only indirect study in which short periods of incubation were employed was reported by Stamm (18), who incubated suspensions of minced calf brain in Locke's solution for periods of 3 to 14 hours and measured the increase in inorganic phosphorus. In nine to fifteen such experiments he found an average increase of 0.13  $\gamma$  per mg of brain at 3 hours, 0.20  $\gamma$  at 4½ hours, and 0.24  $\gamma$  at 6 hours. These values may be compared with the average decrease in lipid phosphorus of 0.19  $\gamma$  per mg of brain found in the 4 hour experiments (Table I) of the present investigation.

In a publication which appeared after most of the experiments listed in Table I had been completed, Fries, Schachner, and Chaikoff (19) reported direct studies, similar to those reported here, on one young (15 gm) and one adult (200 gm) rat. Homogenates of brain tissue in carbonate buffer were incubated for 1, 2, and 4 hours. The quantity of brain per unit volume of solution (about 60 mg per cc) was considerably less than that



employed in most of my experiments and the methods of measurement were also different changes in the quantity of phospholipide were determined by oxidative procedures and by the isotope labeling technique Both methods showed a decrease of 10 to 15 per cent in the phospholipide present

The phospholipide-splitting mechanism of brain appears to be relatively very slow or inoperative in the living animal All available evidence indicates that the rate of turnover of phospholipides in the adult brain is far slower than would be expected if this mechanism were active during life Furthermore, the findings on the brain tissue of young rats cannot be correlated with studies of living animals Fries, Changus, and Chaikoff (4) and Waelsch, Sperry, and Stoyanoff (20) showed that the rate of lipid metabolism in the brain is very rapid during the first few days of life In contrast, Fries, Schachner, and Chaikoff (19) found no difference in phospholipide-splitting ability of brain of the young rat and the adult rat which they studied, and the same result was obtained in the present investigation (*cf* Experiments 33, 34, 35, and 42, Table I, and Experiment 79, Table III)

The author is indebted to Katharina Newerly, David Aaron, and V A Stoyanoff for technical assistance

#### SUMMARY

Homogenates of rat brain tissue in saline-carbonate buffer were incubated at 37.5° with shaking for 4 hours A consistent, though variable decrease, averaging about 8 per cent, in the amount of phosphorus extractable with alcohol-ether took place Increasing the time of incubation up to 24 hours usually increased the magnitude of the change

Extraction with different solvents and with an entirely different technique (11) gave similar results, and rat blood, added in excess, had no consistent effect Total lipides were not changed significantly These findings indicate that the decrease in extractable phosphorus was the result of a cleavage of phospholipides by a mechanism present in brain tissue

Homogenates of brain tissue in the more complex carbonate buffer of Krebs and Henseleit (10), with the phosphate omitted, responded in the same way Results obtained with other buffers were inconclusive No change in extractable phosphorus occurred during incubation of homogenates made in water

Brains of young rats, in which myelination with an active lipid metabolism was going on *in vivo*, showed no more phospholipide cleavage *in vitro* than brains from adult rats

A considerable decrease in alcohol-ether-soluble phosphorus, comparable with that found in homogenates, usually occurred during incubation of minced brain suspended in carbonate buffer

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# THE CONVERSION OF ORNITHINE TO CITRULLINE BY RAT LIVER HOMOGENATES\*

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(Received for publication, June 23, 1947)

The conversion of ornithine to citrulline as an obligatory step in the synthesis of urea in mammalian liver was first postulated by Krebs and Henseleit (1). This reaction was later investigated in detail by Gornall and Hunter (2) using rat liver slices. The use of liver slices for the study of this reaction is unsatisfactory in that citrulline, once formed from ornithine, is rapidly converted through arginine to urea. The use of homogenized liver preparations has eliminated this difficulty in part because of more adequate control of the conditions necessary for the various steps in the cycle.

It was found early in this study that components and conditions necessary for the synthesis of citrulline from ornithine were such as to catalyze the disappearance of citrulline at an appreciable rate when magnesium ions were present (3). The absence of magnesium ions, however, resulted in an accumulation of citrulline. This means of stopping the urea cycle at citrulline provided a convenient system for the study of the optimum conditions necessary for the conversion of ornithine to citrulline.

Srb and Horowitz as a result of studies on *Neurospora* mutants (4) have offered evidence for the existence of two distinct enzymatically catalyzed steps in the conversion of ornithine to citrulline, the first of these being the introduction of carbon dioxide, and the second, the introduction of ammonia. In the present work, this synthesis has been studied as a unit reaction. Experiments reported in this paper deal particularly with the formation of citrulline in the stepwise synthesis of urea from ornithine by rat liver homogenates.

## Procedures

*Preparation of Homogenates*—A detailed description for the preparation of homogenates has been published previously (3). 0.5 ml. of a 20 per cent rat liver homogenate prepared in isotonic KCl was used in each incubation flask throughout the study, unless otherwise specified. This amount of homogenate contained between 2.3 and 2.8 mg. of tissue nitrogen as determined by micro-Kjeldahl analysis.

\* Aided in part by a grant from the Wisconsin Alumni Research Foundation.

*Incubation*—All incubations were carried out in Warburg flasks at 38°, for a period of 1 hour, unless otherwise specified. All flasks were gassed with a 5 per cent CO<sub>2</sub>-95 per cent O<sub>2</sub> mixture previous to introduction into the bath. This procedure resulted in relatively uniform results from experiment to experiment. Further, the gaseous CO<sub>2</sub> present per flask (approximately 1000 microliters) was found to be adequate for the usual length of incubation, as shown by experiments in which regassing at frequent intervals yielded values no higher than those in which the gas had been introduced only once prior to the incubation.

Incubation mixtures giving optimum results consisted of DL-ornithine hydrochloride (at concentrations calculated on the basis of the L isomer only), L-glutamic acid, ammonium chloride, adenylic acid, liver homogenate, potassium phosphate buffer, pH 7.15, and bicarbonate ions at a concentration sufficient to bring the pH to 7.15 after equilibration with 5 per cent CO<sub>2</sub> in the gas phase. The total incubation volume was 3.0 ml.

*Analytical*—At the end of the incubation period, 0.3 ml of 3 M acetate buffer, pH 5.0, was added to stop the reaction. In most experiments, 1.0 ml of incubation mixture was then pipetted into 1.0 ml of 10 per cent trichloroacetic acid and the precipitated protein centrifuged down. 0.5 ml of the clear supernatant was taken for analysis of citrulline by the method of Archibald (5) with slight alterations. In these analyses, the previous destruction of urea by urease was eliminated since the amount of urea formed was negligible. When the amount of urea was measurable, citrulline was determined after a prior treatment with urease and trichloroacetic acid. The period of color development was lengthened to 15 minutes.

For samples to be analyzed for urea, the incubation mixture was centrifuged after the addition of acetate buffer and an aliquot of the supernatant taken for analysis by the method of Krebs and Henseleit (1).

All results are reported in microliters of product, citrulline or urea, or both, per mg of tissue nitrogen. Approximate  $Q_{\text{citrulline}}$  values can be obtained by dividing the microliters of end-product per mg of N by 10, assuming that 10 per cent of the dry weight of liver is nitrogen (6).

*Preparations Used*—DL-Ornithine hydrochloride and DL-citrulline were obtained from the Amino Acid Manufactures, University of California, Los Angeles.

Adenosine triphosphate (ATP) was prepared from rabbit muscle (7) as the hydrated barium salt. Adenylic acid was prepared from ATP by the method of Keil (8). An initial sample of the acid was obtained from Dr. G. A. LePage. The authors are indebted to him for this gift.

$\alpha$ -Ketoglutaric acid and oxalacetic acid were synthetic products (7).

Glutamine samples were obtained from the following sources: Bios Laboratories, Sample A, Dr. F. M. Strong and Dr. R. H. Burns, Samples B

and C respectively, Dr P B Hamilton, Sample D (Lederle), Sample E (synthesized by Dr J S Fruton), and Sample F (Drug Products Company), Dr J P Greenstein, Sample G (Lederle) and isoglutamine. Dr P B Hamilton kindly furnished the assay values for Samples D, E, and F, 82, 97, and 85 per cent purity, respectively. The authors are indebted to the generosity of the above in providing these samples.

All other reagents employed were commercial products.

5 per cent  $\text{CO}_2$ -95 per cent  $\text{O}_2$  and 5 per cent  $\text{CO}_2$ -95 per cent  $\text{N}_2$  gas mixtures were commercial preparations. The same tanks were used throughout the study to insure uniformity of results.

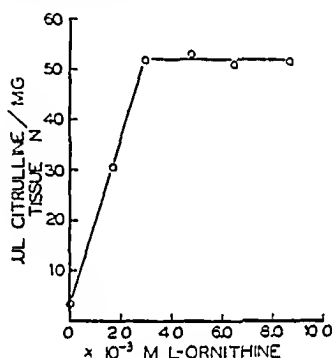


FIG 1

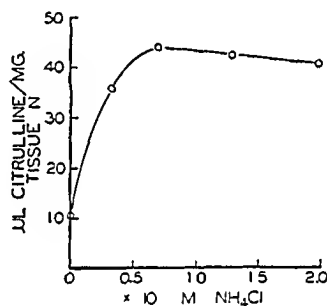


FIG 2

FIG 1 Effect of ornithine concentration on the formation of citrulline. Final substrate concentrations, glutamic acid  $6.7 \times 10^{-2}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M. Tissue concentration per flask 2.64 mg of N.

FIG 2 Effect of ammonia concentration on the formation of citrulline. Final substrate concentrations, glutamic acid  $3.3 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M. Tissue concentration per flask 2.75 mg of N.

### Results

*Substrate Concentrations*—A study of the effect of varying the amount of ornithine revealed the optimum concentration to be 0.0033 M when 2.3 to 2.8 mg of homogenate nitrogen were used per flask (Fig 1). The synthesis of citrulline increased linearly to the optimum level. Further addition of ornithine had no influence on the quantity converted. At 0.0033 final molarity, 50 to 60 per cent of the ornithine was usually converted in 1 hour. This concentration is high compared to the amount of ornithine sufficient to maintain the operation of the complete urea cycle, in which the amino acid is continually regenerated. Preliminary work with homogenates showed that 0.0003 final molarity was as effective with the complete cycle as with any higher concentration.

The effect of increasing concentrations of ammonium chloride is shown

in Fig 2 In the presence of 0.033 to 0.067 M glutamic acid, 0.0067 M ammonium chloride was apparently sufficient to saturate the system

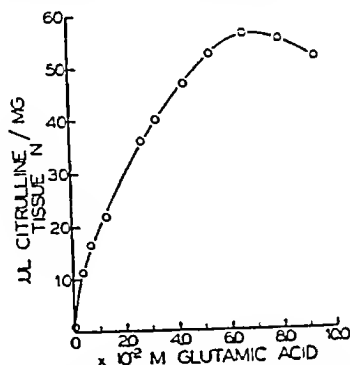


FIG 3 [Effect of glutamic acid concentration on the formation of citrulline Final substrate concentrations, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-2}$  M Tissue concentration per flask 2.64 mg of N

TABLE I

*Percentage of Citrulline Synthesis with Glutamine*

Final substrate concentrations glutamine  $3.3 \times 10^{-2}$  M on the basis of 100 per cent purity (see "Preparations used" under "Procedures" for sources of glutamine samples), glutamic acid  $3.3 \times 10^{-2}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M and  $3.3 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-2}$  M Tissue concentration per flask, 2.45 mg of N

		Relative rate
		per cent
Controls	Glutamic acid $3.3 \times 10^{-2}$ M, ammonium chloride $6.7 \times 10^{-3}$ M	100.0
	Glutamic acid $3.3 \times 10^{-2}$ M, ammonium chloride $3.3 \times 10^{-2}$ M	95.4
Glutamine	Samples A, B, and D	0-0.7
	Sample C	31.7
	" E	27.4
	" F	56.2
	" G	55.2
Isoglutamine		3.9

The optimum glutamic acid concentration was far in excess of the ammonium chloride required (Fig 3). A ratio of glutamic acid to ammonium chloride to ornithine of 15 to 20:2:1 was found to yield optimum values. The peak of the glutamic acid curve was found to vary within 0.045 to 0.067 M concentration from liver to liver, the rising portion of the curve being displaced proportionately. Both glutamic acid and ammonia were

essential, only 15 per cent of the optimum yield being obtained when glutamic acid at  $0.067\text{ M}$  was incubated without ammonium chloride, and 2 to 4 per cent when ammonium chloride at  $0.0067$  to  $0.053\text{ M}$  was used alone. Experiments were carried out at two levels of glutamic acid, 10 and 20 times the concentration of ornithine. While the yield of citrulline increased another 20 per cent at the higher concentration, the trend of the results was identical. Typical data and curves were selected to represent the effect of different substrates on the system in the present paper.

The reason for the requirement of both ammonia and glutamic acid, and particularly of the manifold excess of the latter, is not as yet apparent.

TABLE II

*Citrulline Synthesis with Compounds Other Than Glutamic Acid*

Final substrate concentrations: all compounds listed were present at  $3.3 \times 10^{-3}\text{ M}$ , ornithine  $3.3 \times 10^{-3}\text{ M}$ , adenylic acid  $1 \times 10^{-3}\text{ M}$ , ammonium chloride at  $6.7 \times 10^{-3}\text{ M}$  except with  $\alpha$ -ketoglutaric, malic, fumaric acids, and glucose, for which the relative rates are shown in the order of increasing ammonia concentration from  $6.7 \times 10^{-3}$  to  $3.3 \times 10^{-2}\text{ M}$ . No ammonia was used with asparagine. Tissue concentration per flask: 2.82 mg. of N.

	Relative rate		Relative rate
	per cent		per cent
Glutamic acid	100.0	Fumaric acid	35.4-31.4
Aspartic "	2.4	Succinic "	28.7
Asparagine	2.9	Citric acid	10.9
$\alpha$ -Ketoglutaric acid	21.1-7.8	Lactic "	6.5
Oxalacetic acid	3.5	Glucose	5.3-14.8
Malic acid	26.7-26.2		

Various workers in the field have suggested the importance of glutamine in the synthesis of urea (9, 10). The possibility that glutamine was being synthesized from the glutamic acid and ammonia and entering into the reaction as a more specific donor of ammonia or the carbamino group of citrulline was explored. Experiments were devised in which glutamine replaced ammonium chloride and glutamic acid (Table I). The different samples tested showed a range of activity of 0 to 56 per cent of the control value. A synthetic product (Sample E), assaying 97 per cent pure, was 27.4 per cent as active as glutamic acid plus ammonium chloride. Isoglutamine gave a value of 4 per cent compared with the control.

*Replacement of Glutamic Acid*—Of a series of compounds tested in place of glutamic acid none showed more than 35 per cent of the activity of glutamic acid (Table II). Previous work with liver slices had shown a stimulation of urea synthesis by many of the components listed in a system in-

volving added ornithine and ammonia (1, 2, 10). However, in the case of homogenates, glutamic acid appears to have a relatively specific effect. The analogue, aspartic acid, and asparagine, its half amide, were inactive. As can be seen from Table II, the main components of the citric acid cycle

TABLE III

*Effect of Partial Replacement of Glutamic Acid with Other Compounds*

Final substrate concentrations: ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M. Tissue concentration per flask, 2.53 mg of N.

	Citrulline per mg tissue %	
	microliters	
Glutamic acid 0.013 M	37.8	
" " 0.026 "	47.1	
" " 0.013 " + fumaric acid 0.013 M	34.6	
" " 0.013 " + succinic " 0.013 "	33.2	
" " 0.013 " + $\alpha$ -ketoglutaric acid 0.013 M	41.4	
" " 0.013 " + oxalacetic acid 0.013 M	43.6	
" " 0.013 " + lactic acid 0.013 M	38.8	
" " 0.013 " + citric " 0.013 "	39.2	

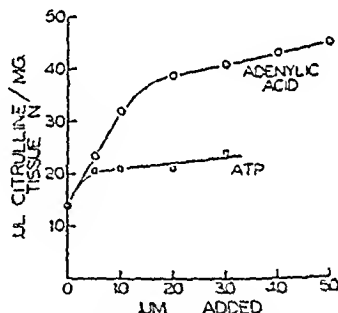


FIG. 4. Effect of adenylic acid and ATP concentrations on the formation of citrulline. Final substrate concentrations, glutamic acid  $3.3 \times 10^{-3}$  M, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M. Tissue concentration per flask 3.24 mg of N.

showed values ranging from 3.5 per cent activity with oxalacetic acid to 35 per cent with fumaric acid. A few of these compounds were tested through a varying range of ammonium chloride concentration (0.0067 to 0.033 M).  $\alpha$ -Ketoglutaric acid, malic acid, and fumaric acid showed a decrease in activity with an increase in ammonia. Glucose activity, on the other hand, increased with the ammonia concentration.

The presence of other compounds in addition to a suboptimum concentration of glutamic acid (0.013 M) was in most cases no more effective than



glutamic acid alone (Table III). Fumaric acid and succinic acid decreased the yield of citrulline, but citric acid and lactic acid had no significant effect. While  $\alpha$ -ketoglutaric acid and oxalacetic acid increased citrulline formation, they were only 38.7 and 62.4 per cent effective as an equal additional quantity of glutamic acid.

**ATP and Adenylic Acid**—Both ATP and adenylic acid stimulated the synthesis of citrulline (Fig. 4). Results obtained with ATP varied from liver to liver within 50 per cent of the values shown. In all probability its effect in this system is dependent on its prior hydrolysis to the more

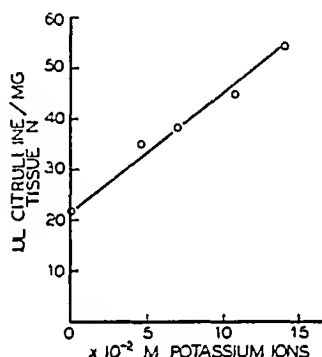


FIG 5

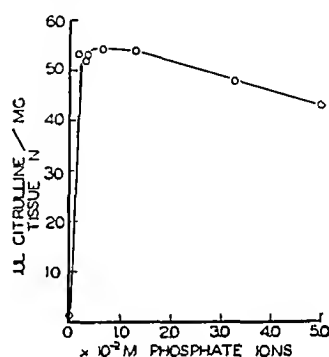


FIG 6

FIG 5 Effect of potassium ion concentration on the formation of citrulline. Final substrate concentrations, glutamic acid  $6.7 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M. Tissue concentration per flask 3.22 mg of N.

FIG 6 Effect of phosphate ion concentration on the formation of citrulline. Final substrate concentrations, glutamic acid  $6.7 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M. Tissue concentration per flask 3.01 mg of N.

active adenylic acid. The fact that the latter reagent gave more consistent results at higher levels would indicate that it, *per se* or through the formation of some intermediate other than ATP, is the more active component in this synthesis. 3 micromoles of adenylic acid were used per flask throughout the study.

**Cytochrome c**—Since cytochrome *c* stimulated the synthesis only 7 per cent at the relatively high concentration of 0.000035 M, it was not used in subsequent experiments.

**Oxygen and Carbon Dioxide**—No synthesis of citrulline from ornithine occurred anaerobically. Bicarbonate ions and gaseous  $\text{CO}_2$  were used in all experiments throughout the study. The elimination of either added bicarbonate or gaseous  $\text{CO}_2$  from the incubation flask resulted in about 50 per cent as much conversion as with the complete system.

*Kidney Homogenate*—No synthesis of citrulline was noted when kidney homogenate was incubated under the conditions found optimum for liver homogenate

*Inorganic Ions*—Stimulation by potassium ions increased linearly to the maximum possible concentration (Fig 5) 250 per cent as much activity was seen when all the sodium ions, except that added as sodium bicarbonate, had been replaced by potassium ions Since commercial potassium bicarbonate contains magnesium ions as an impurity, it was not used in this study

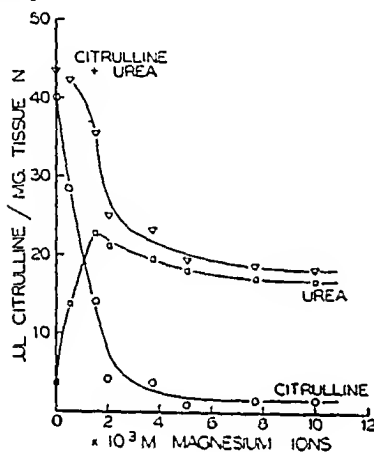


FIG 7

FIG 7 Effect of magnesium ion concentration on the formation of citrulline and urea Final substrate concentrations, glutamic acid  $3.3 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M Tissue concentration per flask 3.45 mg of N

FIG 8 Effect of pH on the formation of citrulline Final substrate concentrations, glutamic acid  $3.3 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M Tissue concentration per flask 2.8 mg of N

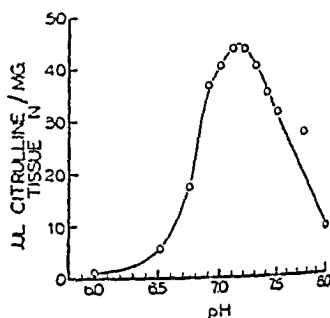


FIG 8

Stimulation was also noted with phosphate ions (Fig 6) Essentially no synthesis occurred in the absence of these ions Maximum activity is reached at relatively low concentrations, 0.002 to 0.015 M This latter molarity, added in the form of phosphate buffer, was used throughout the study Higher concentrations inhibited the synthesis The influence of phosphate ions suggests a specific effect, since concentrations of 0.002 M phosphate are too low to exert a significant buffering action in this system

The effect of magnesium ions on the synthesis of citrulline and urea is seen in Fig 7 The absence of these ions resulted in the accumulation of citrulline With an increase in concentration to 0.0015 M, more of the citrulline formed was converted to urea Higher concentrations appear to inhibit both reactions In previous work (3), the effect of magnesium ions

on the conversion of citrulline to arginine was interpreted on the basis of its reported influence on ATP breakdown (11). Data presented here point to a more specific rôle of magnesium ions in that reaction. The possibility that citrulline accumulated as a result of stopping the urea cycle at arginine was investigated, since arginase requires covalent ions for activation. No arginine was found. Moreover, in the absence of any added ion, 0.0033 M arginine was found to be converted almost completely to urea in an hour by liver homogenate.

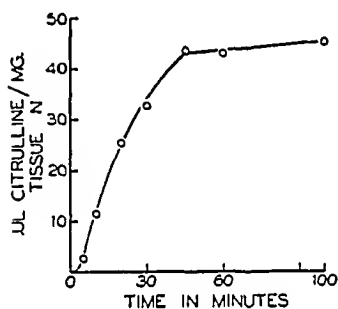


Fig 9

FIG 9 Effect of incubation time on the formation of citrulline. Final substrate concentrations, glutamic acid  $3.3 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M. Tissue concentration per flask 2.53 mg of N.

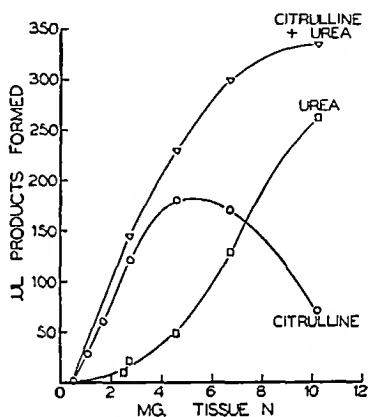


Fig 10

FIG 10 Effect of tissue concentration on the formation of citrulline and urea. Final substrate concentrations, glutamic acid  $3.3 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M. Tissue concentration per flask 2.81 mg of N.

Manganese ions below 0.001 M exerted approximately the same effect as magnesium ions.

**Effect of pH**—The range between pH 7.1 to 7.2 was optimum for the synthesis of citrulline (Fig 8). Both bicarbonate-carbon dioxide and phosphate buffers were used to maintain pH. The concentrations of bicarbonate to be added to the media were calculated from the Henderson-Hasselbalch equation on the basis of a 5 per cent  $\text{CO}_2$  content in the gas phase. At the optimum pH of 7.15, the concentration of the bicarbonate added to the media was 0.008 M.

**Incubation Time**—Production of citrulline usually slowed down abruptly after 50 to 60 minutes of incubation (Fig 9). At this time, approximately 50 to 60 per cent of the ornithine added was found to be converted. The

reason for this abrupt decrease in the rate of synthesis is not clear. No citrulline disappeared within that time, nor was there any significant amount of urea formed. The possibility exists that ornithine is being removed in this system by some other reaction.

*Tissue Concentration*—In the absence of magnesium ions, citrulline accumulated in incubation flasks containing from 0.5 to 4 mg of tissue nitrogen (Fig. 10). The conversion of citrulline to urea in this range of tissue concentration was very small. As the tissue concentration was increased, less citrulline and more urea were formed. The shape of these curves suggests two possibilities: (1) that either the presence of urea inhibits the formation of citrulline, or (2) that a protein-bivalent ion complex catalyzes the disappearance of the amino acid. The former possibility was eliminated.

TABLE IV

*Effect of Inhibitors on Synthesis of Citrulline*

Final substrate concentrations: glutamic acid  $6.7 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-2}$  M, ammonium chloride  $6.7 \times 10^{-2}$  M, adenylic acid  $1 \times 10^{-2}$  M. Tissue concentration per flask, 2.81 mg of N.

Inhibitors	Final molarity	Per cent inhibition
Arsenite	0.001	97.0
Cyanide	0.001	96.1
Iodoacetate	0.01	72.5
Azide	0.001	14.8
Malonate	0.003	15.7
"	0.0057	58.8
Calcium ions	0.001	98.6

through experiments which showed that urea had no inhibiting effect. The existence of a magnesium-activated enzyme catalyzing the conversion of citrulline to arginine, on the other hand, is not unlikely. The fact that there is little citrulline converted at low tissue concentrations in the absence of magnesium ions indicates that the ion-protein complex exists in favor of the inactive dissociated form. Thus an increase in homogenate nitrogen per flask correspondingly increases the concentration of the combined form to a point at which the rate of citrulline conversion exceeds the rate of its formation. In the presence of magnesium ions no citrulline accumulated at any tissue nitrogen level.

*Inhibitors*—Of a group of various inhibitors tried (Table IV), arsenite, cyanide, and calcium ions inhibited almost completely at 0.001 M. Since these substances are known to affect oxidative mechanisms, their effect in this system was not unexpected.

Of interest was the finding that fluoride stimulated the synthesis of

citrulline in concentrations up to 0.007 M (Fig. 11) 0.05 M was required for almost complete inhibition. In previous work, fluoride was found to inhibit the conversion of citrulline to arginine almost completely at 0.01 M (3). This differential inhibition by fluoride of the two reactions can also be used to advantage in studying the synthesis of citrulline.

The magnitude of inhibition seen with the other substances listed in Table IV is similar to that observed with the transamination reaction with the exception of malonate which is less effective in this system. Studies on

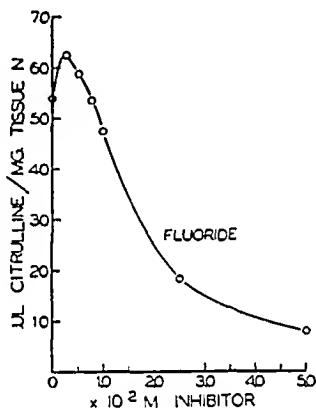


FIG 11

FIG 11 Effect of fluoride concentration on the formation of citrulline. Final substrate concentrations, glutamic acid  $6.7 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M. Tissue concentration per flask 3.22 mg of N.

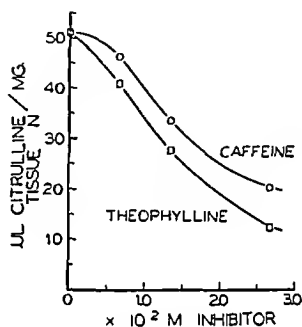


FIG 12

FIG 12 Effect of caffeine and theophylline on the formation of citrulline. Final substrate concentrations, glutamic acid  $6.7 \times 10^{-2}$  M, ornithine  $3.3 \times 10^{-3}$  M, ammonium chloride  $6.7 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M. Tissue concentration per flask 2.11 mg of N.

caffeine inhibition of urea synthesis have been carried out by Bernheim and Bernheim (12). They found that caffeine and like derivatives inhibited the disappearance of ammonia in liver and kidney slices and the synthesis of urea in liver slices and in man *in vivo*. A study of a series of purines revealed that caffeine and theophylline inhibited the synthesis of citrulline from ornithine and not at all the transamination reaction. Inhibition by theophylline was more marked than by caffeine (Fig. 12).

#### DISCUSSION

On the basis of the present data and those previously reported (3, 13), it is apparent that the enzymatic steps in the synthesis of urea by mammalian liver can be studied in properly fortified cell-free systems. The

need for coupling with some energy-yielding mechanism is indicated from these studies. The greater effectiveness of adenylic acid over that of ATP in the conversion of ornithine to citrulline would suggest either a specific effect of adenylic acid or the mediation of an energy-coupling reaction which does not involve ATP as such.

The apparently obligatory rôle of glutamic acid in both the conversion of ornithine to citrulline and of citrulline to arginine is of particular interest. This key rôle of glutamic acid in the urea cycle again supports the unique position of this amino acid in intermediary metabolism. The recent finding of Krebs, Eggleston, and Hems (14) that liver homogenates are capable of rapidly forming glutamic acid from  $\alpha$ -ketoglutaric acid and ammonia under anaerobic conditions provides a mechanism for supplying glutamic acid from the intermediary metabolic pool of nitrogen compounds for the transamination reaction as well as for the conversion of ornithine to citrulline. It is of interest to point out that any amino acid capable of transamination with  $\alpha$ -ketoglutaric acid could contribute its nitrogen to urea without having to undergo primary oxidative deamination.

While the rôle of glutamic acid in the transamination reaction is clear, its rôle in the conversion of ornithine to citrulline is obscure. The need for concentrations of glutamic acid 10 to 20 times that of ornithine or ammonia does not permit any simple stoichiometric relationship. Further, the requirement for ammonium ions in the presence of high glutamic acid concentrations makes the possibility of direct amino group transfer from glutamic acid unlikely. The possible rôle of glutamine in this system would seem to be excluded from the data presented. Another possibility is that glutamic acid is required as a cometabolite for an energy-yielding system coupled with the synthesis of citrulline from ornithine. This is somewhat difficult to accept in view of the specificity for glutamic acid in this system. More direct information on this point is anticipated from projected studies with isotopically labeled glutamic acid.

It should be pointed out that the system reported here capable of synthesizing citrulline from ornithine, carbon dioxide, and ammonia has been considered as a single enzymatic step. It is highly probable that at least two reactions are involved in this synthesis and consequently an analysis of the rôle of the various reactants will be difficult until these enzymatic steps are resolved.

#### SUMMARY

1. The conversion of ornithine to citrulline has been demonstrated to occur in rat liver homogenates in the presence of glutamic acid, ammonium ions, adenylic acid or adenosine triphosphate, phosphate, and bicarbonate carbon dioxide buffers. ATP was found to be 50 per cent as effective as adenylic acid.

2 Components of the citric acid cycle and other compounds similar to glutamic acid showed only up to 56 per cent as much activity as glutamic acid when substituted in part or completely for the acid

3 The formation of citrulline is stimulated by phosphate and potassium ions

4 The reaction proceeded maximally at a pH of 7.15

5 In the presence of magnesium ions and high homogenate tissue concentrations, the citrulline formed was converted to urea

6 Fluoride stimulated the synthesis up to 0.007 M concentration, complete inhibition occurred at 0.05 M. Caffeine and theophylline progressively inhibited the reaction with increasing concentrations

7 The significance of these findings in relation to the Krebs-Henseleit urea cycle is discussed

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# A SURVEY OF TRANSAMINASES IN PLANTS\*

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(Received for publication, July 14, 1947)

The transamination reaction of Braunstein and Kritzman (1) has been studied in green plant tissue by Virtanen and Laine (2), Cedrangolo and Carandante (3), Albaum and Cohen (4), and Rautanen (5). Since few data have been presented on the distribution of the transaminase enzymes in various plant tissues and kinds of plants, more data are necessary in order to evaluate the role of the transamination reaction in protein metabolism.

The occurrence of the aspartic-glutamic transaminase was investigated in the parts of twenty-two plant species at various ages.

## Methods

*Preparation of Enzyme Extract*—Extracts were prepared by comminution of plant tissue with a Nixtamal mill, by hand grinding with sand, or with a glass homogenizer (6). Samples were ice-cooled either before or immediately after grinding. Extracts were adjusted to pH 8.0 with the aid of a glass electrode. All preparations were centrifuged in a Beams air-driven, "spinning top" centrifuge (7) and the supernatant was employed. Total nitrogen of homogenates and saps was determined by the semimicro-Kjeldahl method (8).

The embryos used in the studies were prepared by sterilizing seeds with 1:1000 HgCl<sub>2</sub>. The seeds were soaked in sterile distilled water on blotting paper in Petri dishes and then germinated by incubation in a moist chamber in the dark at 30°. Barley was grown under controlled conditions of moisture and temperature in a malting chamber. Embryos were carefully dissected from the seeds and homogenized in ice-cold 0.1 M phosphate buffer (pH 8.0). Unheated wheat germ (2 per cent by weight in buffer solution) was likewise homogenized.

The 2, 4, and 6 week-old plants used in the experiments were grown in a greenhouse in sand supplied with nutrient solution. In sampling, petioles were treated as stem tissue. Leaf tissue included only the leaf blade.

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

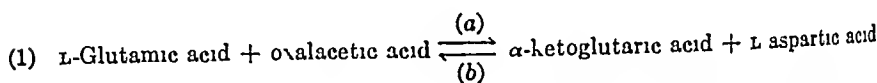
After grinding, the sap was expressed through cheese-cloth and centrifuged. In almost all experiments the undiluted sap was used.

*Analytical Methods*—Glutamic acid was determined with a bacterial decarboxylase as described by Umbreit and Gunsalus (9). Oxalacetic acid was determined by the aniline citrate method (10).

*Substrates*—Oxalacetic acid was prepared from sodium diethyl oxalacetate (10), and the  $\alpha$ -ketoglutaric acid was kindly supplied by Dr F H Stodola (11). Merck amino acids were used.

### Procedure

The following transamination reaction was studied



The forward reaction, Reaction 1, *a*, was carried out in 18 × 150 mm test-tubes which were attached to a mechanical shaker and immersed in a water bath maintained at 30°. No attempt was made to provide anaerobic conditions. A 4 to 5 ml sample of the transaminase preparation (pH 8.0) was incubated with 2.0 ml of 0.06 M oxalacetic acid for 10 minutes. Then 2 ml of 0.06 M glutamic acid (pH 8.0) were added and the reaction allowed to proceed for various incubation periods. The reaction was stopped by the addition of 1.0 ml of 10 per cent sulfuric acid. Controls containing oxalacetic acid and enzyme and controls containing glutamic acid and enzyme to test recovery were included. In order to decompose any remaining oxalacetic acid, the contents of the tubes were adjusted to pH 2.0 and were immersed in a boiling water bath for 1 hour. After cooling, the samples were carefully adjusted to pH 5.0, transferred to 10 ml volumetric flasks, and made up to volume. Each sample was then centrifuged and a 2.0 ml aliquot was placed in a Warburg flask for analysis. After a 10 minute equilibration period at 30°, 0.5 ml (10 mg) of glutamic acid decarboxylase preparation (9), suspended in potassium acid phthalate buffer (pH 5.0), was added from the side arm and evolution of CO<sub>2</sub> was measured.

For measuring the reverse reaction, Reaction 1, *b*, the enzyme preparation and 0.5 ml of 0.03 M DL-aspartic acid (pH 8.0) were placed in the main compartment of the Warburg flask. In one arm 0.5 ml of 0.03 M  $\alpha$ -ketoglutaric acid (pH 8.0) was placed, and in the other side arm 0.5 ml of aniline citrate (equal volumes of 50 per cent citric acid and freshly distilled aniline). After 10 minutes equilibration at 30°, the  $\alpha$ -ketoglutaric acid was mixed with the sample and the transamination reaction allowed to proceed. The reaction was stopped and analyzed by adding the aniline citrate. The decomposition of the oxalacetic acid formed in the transami-

nation reaction was essentially complete in 10 minutes. Controls with  $\alpha$ -ketoglutaric acid omitted were included.

### Results

*Germinating Seed Embryos*—Table I presents data on the ability of germinating seed embryo homogenates to catalyze Reactions 1, *a* and 1, *b*.

TABLE I  
*Transamination in 48 Hour-Old Germinating Seed Embryos*

Germinating seed	Transamination period	Reaction 1, <i>a</i>			Reaction 1, <i>b</i>		
		Control recovery (glutamic acid + enzyme)*	Transamination†	Q <sub>T</sub> (N)‡	Oxalacetic acid formed	Transamination§	Q <sub>T</sub> (N)‡
	<i>min</i>	<i>per cent</i>	<i>per cent</i>		<i>micromoles</i>	<i>per cent</i>	
Green beans	5				1.36	9.1	21.6
	10	82	32.4	75.2	1.86	12.5	14.5
Sweet corn	5				2.19	14.7	46.0
	10	86	47.8	116.0	2.28	15.5	24.3
Garden peas	5				0.29	2.0	6.2
	10	86	29.6	83.6	0.69	4.6	7.4
Radish	5				2.24	15.0	32.8
	10	85	33.8	73.8	2.56	17.2	18.8
Cucumber	5				1.04	7.0	19.7
	10	86	21.5	61.1	1.22	8.2	11.6
Clover	5				1.66	11.2	8.1
	10	83	32.9	38.2			
Soy beans	5						
	10	83	32.9	38.2	1.66	11.2	8.1
Vieland oats	5						
	10		45.3	188.7			

\* The percentage of initial glutamic acid (24 micromoles in the aliquot assayed, equivalent to 538 microliters of CO<sub>2</sub> on decarboxylation) added to the controls which remained at the end of the transamination period.

† The percentage of initial glutamic acid which was consumed in Reaction 1, *a*.

‡ Q<sub>T</sub> (N) = (micromoles of amino acid transaminated)/(mg of N × hours).

§ Percentage of initial added aspartic acid which was transaminated, as measured by oxalacetic acid formation.

In the controls for Reaction 1, *a*, in which glutamic acid and the enzyme preparation were incubated (no oxalacetic acid added) and the amount of glutamic acid then determined, a small amount of the initial glutamic acid disappeared. Since this amount may easily have been consumed by means of the transamination reaction, it was decided to base percentage transamination on the initial quantity of glutamic acid present. The percentages of transamination in 10 minutes for Reaction 1, *b* are one-half to one-third those for Reaction 1, *a*. This ratio agrees with that recorded in the literature for oat seedlings (4).

TABLE II

*Transamination in Barley, Reaction 1, b*

Malting temperature, 15-16°

Variety	Age	Transamination period	Transamination	Qt(N)
	<i>hrs</i>	<i>min</i>	<i>per cent</i>	
Reno	24	5	9.6	59.2
		10	13.1	40.4
		15	17.4	35.8
Manchuria	24	5	9.5	48.6
		10	13.3	33.8
		15	16.7	28.5
Korsbyg	24	5	11.8	41.3
		10	13.1	22.9
Kindred	24	5	12.4	43.4
		10	13.3	23.3
Reno	72	5	8.9	72.0
		10	12.3	50.1
		15	14.9	40.3
Manchuria	72	5	9.9	70.8
		10	11.9	42.8
		15	15.8	36.5
Korsbyg	72	5	8.0	27.5
		10	10.5	18.6
Kindred	72	5	9.9	34.0
		10	12.1	20.8
Reno	120	5	9.5	72.7
		10	9.8	38.0
		15	14.2	36.8
Manchuria	120	5	8.2	46.0
		10	13.8	38.5
		15	13.9	25.3
Korsbyg	120	5	8.9	32.5
		10	12.9	23.6
Kindred	120	5	11.1	36.9
		10	14.0	23.1
	Moisture		Nitrogen (dry weight basis)	
	<i>per cent</i>		<i>per cent</i>	
Reno	47.2		2.49	
Manchuria	45.4		2.43	
Korsbyg	48.5		2.23	
Kindred	48.5		2.28	

Reaction 1, b was studied in several varieties of barley at various stages of malting. The values obtained are recorded in Table II. Two varieties,

Reno and Manchuria, have a high nitrogen content and germinate rapidly. Both germinated while being steeped and were quite advanced at the 24 hour stage. Koisbyg and Kindred, on the other hand, have a lower nitrogen content and germinate slowly. The faster germinating barley had the higher transaminase activity,  $Q_T(N)^1$  values at all stages were much higher than those for the more slowly germinating barley. It is of interest to note the sharp fall of transaminase activity of 72 hour-old embryos of the Koisbyg and Kindred varieties.  $Q_T(N)$  values were high at 24 hours, decreased at 72 hours, and then increased again at 120 hours. These temporal changes did not occur with the Reno and Manchuria varieties. The competition of other enzymatic systems is probably more in evidence in the slower germinating barley, Koisbyg and Kindred, than in the faster germinating barley.

TABLE III  
*Transamination in Wheat Germ*

Transamination period	Reaction 1, <i>a</i>			Reaction 1, <i>b</i>		
	Glutamic acid transaminated	Transamination	$Q_T(N)$	Oxalacetic acid formed	Transamination	$Q_T(N)$
<i>min</i>	<i>micromoles</i>	<i>per cent</i>		<i>micromoles</i>	<i>per cent</i>	
5	5.22	21.7	73.6	1.56	10.5	24.0
10	7.14	29.7	52.6	2.09	14.0	16.0
15	8.12	33.8	39.8	2.60	17.3	13.3
20	9.81	40.8	36.2	2.42	16.2	

*Wheat Germ*—The transaminase activity of commercial unheated wheat germ was investigated, its use was suggested to us by Dr. P. P. Cohen who had found it a convenient material with high transaminase activity. Table III presents data obtained for Reactions 1, *a* and 1, *b*. Examination of the percentages of transamination for the different periods of time reveals that Reaction 1, *a* is about 3 times as fast as Reaction 1, *b*. This ratio again agrees with that recorded in the literature (4).

Wheat germ preparations were also tested for their ability to transfer the amino group from alanine to  $\alpha$ -ketoglutarate. This transamination, as measured by pyruvate formation, occurred at a rate comparable to that of Reaction 1 and gave a  $Q_T(N)$  of 40.6 over a 10 minute period.

*Young and Mature Plant Tissue*—The distribution of glutamic-aspartic transaminase was studied in a number of young and mature plants. Values

<sup>1</sup>  $Q_T(N) = (\text{micromoles of amino acid transaminated}) / (\text{mg of N} \times \text{hours})$ . This expression of transamination on a micromolar basis when multiplied by 22.412 is equivalent to the  $Q_{TV}$  on an equivalent  $\text{CO}_2$  basis as used by Lichstein and Cohen (12).

TABLE IV  
Transamination in Plant Tissue, Reaction 1, b

Plant	Age	Tissue	Transami- nation period	Oxalacetic acid formed	Transami- nation	Qr (%)
	<i>wks</i>		<i>min</i>	<i>micromoles</i>	<i>per cent</i>	
Soy bean	2	Leaf	10	3.70	24.7	8.6
		Stem	10	2.64	17.6	13.8
		Root	10	2.40	16.1	47.7
	6	Leaf	10	1.91	12.8	5.9
		Stem	10	2.22	14.8	8.1
		Root	10	2.15	14.4	40.3
	10	Nodules	5	1.73	11.6	24.3
			10	2.60	17.4	18.3
Potato	2	Leaf	10	1.98	13.4	5.3
		Stem	10	0.88	5.9	11.1
		Root	10	0.96	6.5	14.4
	6	Leaf	10	3.30	22.1	6.1
		Stem	10	0.96	6.5	13.2
		Root	10	1.58	10.6	25.7
Tomato	2	Leaf	10	2.62	17.6	16.1
		Stem	10	2.53	17.0	36.0
		Root	10	1.54	10.3	82.0
	6	Leaf	10	1.78	11.9	8.1
		Stem	10	0.73	4.9	7.3
		Root	10	2.00	13.4	19.4
Beet	2	Leaf	10	1.71	11.5	8.4
		Stem	10	0.78	5.2	38.8
		Root	10	0.41	2.8	17.6
	10	Leaf + stem	10	2.54	17.0	8.0
		Root	10	2.60	17.4	8.5
Lettuce	2	Leaf + stem	10	1.73	11.6	23.1
		Root	10	0.82	5.5	24.6
Cabbage	2	Leaf	10	2.24	15.0	15.6
		Stem	10	1.21	8.1	48.3
		Root	10	0.57	3.8	10.0
Barley, Manchuria	2	Leaf + stem	10	2.00	13.4	9.0
“ Korsbyg	2	“ + “	10	2.22	14.9	9.1
		Root	10	0.93	6.3	18.6
Carrot	10	Leaf	10	0.59	3.9	3.8
		Root	10	2.79	18.7	52.2
Squash	10	Fruit	10	2.13	14.3	7.6
Green tomatoes		“	10	0.35	2.3	
“ apples		“	10	0.00	0.0	0.0
Garden peas	12	Leaf	10	1.53	10.2	3.2
		Stem	10	2.41	16.1	3.5
		Pods	5	2.42	16.2	16.3
			10	3.57	23.9	12.1

TABLE IV—*Concluded*

Plant	Age	Tissue	Transami- nation period	Oxalacetic acid formed	Transmi- nation	2
	<i>wks</i>		<i>min</i>	<i>micromoles</i>	<i>per cent</i>	
Kohlrabi	12	Leaf	10	1 07	7 1	3 9
		Stem	10	2 29	15 4	16 6
Green beans	9	Leaf	10	1 84	12 4	6 4
		Stem	10	3 07	20 6	9 7
		Root	10	1 86	12 4	22 2
		Nodules	10	2 33	15 6	19 7
Maple		Leaf	10	0 40	2 7	6 0
Pine		Needles	10	0 00	0 0	0 0

TABLE V

*Transamination in Plant Tissue, Reaction 1, a, 10 Minutes*

Plant	Age	Tissue	Control recovery (glutamic acid + enzyme)	Glutamic acid transami- nated	Transami- nation	Q <sub>T</sub> (N)
	<i>wks</i>		<i>per cent</i>	<i>micromoles</i>	<i>per cent</i>	
Soy bean	10	Nodules	80	9 32	39 1	82 0
Potato	2	Root	81	7 85	32 9	146 3
		Stem	89	6 51	27 3	102 2
		Leaf	86	8 70	36 5	29 0
Tomato	6	Leaf		6 25	26 2	35 2
		Root		9 24	38 7	111 0
Garden peas	12	Pods	82	10 00	41 8	33 7
Squash	10	Leaf	80	13 47	56 5	45 5
Barley, Korsbyg	2	"	89	7 90	33 1	44 2

for Reaction 1, *b* are presented in Table IV. Soy beans, potatoes, tomatoes, and beets were investigated at the 2 week stage and again at the 6 or 10 week stage. Transamination ability tended to decrease with age in soy beans, tomatoes, and beets, but 6 week-old potato plants showed a slightly greater activity than the 2 week-old plants. The greatest activity, expressed on a nitrogen basis, was always found in the roots (except beet and cabbage). In all examples cited greater activity was found in the leaves of the 2 week-old plants than in the leaves of mature plants (except potato). Carrot root had an abnormally high  $Q_T(N)$ . Roots and nodules of green beans were more active than the leaves or stems. The active nitrogen metabolism in green pea pods probably accounted for the high values in the pods, in contrast to the lower values found in the other tissues of the pea. The fleshy stem of kohlrabi had high activity and this was also true of 2

week-old cabbage stems The presence of transaminase in squash fruit was of interest The enzyme could not be detected in any appreciable amounts in such fruits as green apples or green tomatoes

Reaction 1, *a* was also studied in various plant tissues The results are presented in Table V Greatest activity, on a nitrogen basis, is again exhibited by the roots

#### DISCUSSION

Transaminase activity was demonstrated in various tissues from many types of plants Activity was always found in tissues containing significant quantities of crude protein, but in tissues low in protein, such as green apples, green tomatoes, maple leaves, and pine needles, marked activity could not be demonstrated Although mature plants generally have a less intense protein metabolism than germinating seeds, their tissues showed high transaminase activity The  $Q_T(N)$  values for many mature tissues were of the same order of magnitude as for germinating seeds Leaves had active systems, showing a higher percentage transamination of substrate in a given length of time per ml of sap than did either roots or stems However, a more valid comparison can be made from the  $Q_T(N)$  values which are based on the nitrogen content of the enzyme preparations, roots, with a low nitrogen content, showed higher  $Q_T(N)$  values than leaves In view of the general occurrence and high activity of transaminase in plant tissues, it seems probable that transamination functions actively in the protein metabolism of plants

#### SUMMARY

1 Embryos of germinating seeds were tested for their ability to catalyze the forward and reverse glutamic-aspartic transamination reaction In the case of malt sprouts the transamination quotient tended to increase with age and leveled off at the 120 hour stage

2 Very active transaminases, catalyzing both the glutamic-aspartic and glutamic-alanine transaminations, were found in unheated wheat germ

3 The presence and distribution of aspartic-glutamic transaminase in mature higher plants was demonstrated Leaf, stem, root, fruit, and nodular tissue all catalyzed the reaction Leaf tissue was found to be very active per unit volume of sap, but roots and nodules had higher  $Q_T(N)$  values

4 The transamination rate per unit of tissue decreased with age in the growing plant At maturity the  $Q_T(N)$  values for the various tissues were markedly lower than for the 2 week-old plant tissues

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# THE ENZYMATIC DEGRADATION OF COZYMASE AND THE INHIBITORY ACTION OF $\alpha$ -TOCOPHERYL PHOSPHATE

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(Received for publication, July 14, 1947)

A cozymase nucleotidase, the activity of which is inhibited by nicotinamide, was postulated by Mann and Quastel (1) as existing in rat brain, kidney, and liver. Later, Handler and Klein (2) made a study of the end-products of pyridine nucleotide breakdown by animal tissues and found that the first product to be formed is nicotinamide. Since the dinucleotide is split at a typical nucleosidic linkage and phosphate is not liberated primarily, the enzyme concerned in this paper will be referred to as a nucleosidase.

Houchin (3) reported that  $\alpha$ -tocopheryl phosphate ( $\alpha$ -TPh) lowers toward normal levels the high rate of oxygen consumption of dystrophic muscle slices from rabbits and hamsters. This decrease he concluded was accomplished through a suppression of the succinoxidase activity of the dystrophic muscle. Recently, Basinski and Hummel (4) have shown that, contrary to Houchin's findings, the succinoxidase activity of dystrophic muscle is not abnormally high. This, however, does not negate the possibility that the lowering of the  $QO_2$  of dystrophic muscle tissue by added  $\alpha$ -TPh may be due, in part, to a suppression of succinoxidase activity.

Govier *et al* (5) have reported marked inhibition of the succinoxidase system of guinea pig heart by  $\alpha$ -TPh. More recently, working with tissues from vitamin E-deficient animals, he has shown (6) that either  $\alpha$ -TPh or digitoxin greatly increases the inhibition of the succinodehydrogenase system caused by diphosphopyridine nucleotide (DPN). He postulated the following mechanism as an explanation of this effect:  $\alpha$ -TPh inhibits DPN-nucleosidase, thereby helping to preserve DPN. In turn, DPN aids in the conversion of malate to oxalacetate, which is a potent antagonist of the system succinate  $\xrightarrow{\text{succinodehydrogenase}}$  fumarate.

It is the purpose of this paper to report some more direct studies on the enzyme system in animal tissues, which *in vitro* causes the liberation of nicotinamide from DPN. Particular attention has been accorded the effect of  $\alpha$ -TPh on the system; digitoxin, inositol, and calcium ions have also been tested for possible inhibition or stimulation of nucleosidase activity.

### Materials

DPN, prepared from yeast, was obtained commercially<sup>1</sup>. The purity of the material was determined by ultraviolet absorption measurements (7) in the Beckman spectrophotometer and the manufacturer's statement of DPN content was confirmed. All of the DPN was present in the oxidized form.

Disodium  $\alpha$ -TPh used in these experiments was a synthetic preparation<sup>2</sup>.

Albino, Wistar strain rats of either sex were employed as the tissue source. These animals had been maintained on a diet of "master dog cubes" (Toronto Elevators). The tissue samples were homogenized in 0.9 per cent saline and centrifuged, the residue was washed twice in the centrifuge tube with fresh saline and was finally diluted to a suitable volume with 0.02 M phosphate buffer at pH 7.2.

### Methods

Test reactions were carried out in 15 ml centrifuge tubes, each of which, unless otherwise specified, contained 0.5 ml of a solution containing 12 micromoles of pure DPN, 0.5 ml of a solution of  $\alpha$ -TPh or other substance under test, and 1.0 ml of washed tissue homogenate diluted with phosphate buffer. In the control tubes, distilled water replaced the suspected inhibitor. The tissue homogenate was added by pipette after the tubes and solutions had been equilibrated in the water bath at 37°. The total volume of the test mixture was 2.0 ml.

After digestion for 20 minutes at 37° the reaction was stopped and the protein precipitated by the addition, with thorough mixing, of 1.0 ml of 0.11 M zinc sulfate solution followed by 1.0 ml of a 0.1 N solution of sodium hydroxide. After centrifuging, 2.0 ml of the supernatant fluid were removed by pipette and the nicotinamide content was determined in the Coleman spectrophotometer according to the method of Melnick and Field (8). In applying this method it was found that the color which developed on the addition of the aniline reached its maximum intensity in 5 to 10 minutes and deviated from the maximum by less than 3 per cent during the following 30 minutes. Readings of the test solutions were made at the convenient interval of 30 minutes after the aniline was added. It was necessary to prepare a standard curve with each series of determinations, since it was found that the position of the curve fluctuated, while its shape remained relatively constant.

<sup>1</sup> Obtained from the Schwarz Laboratories, Inc., 202 East 44th Street, New York. Lot 1 contained 40 per cent DPN and Lot 2 contained 60 per cent DPN.

<sup>2</sup> This material was kindly supplied by Dr. J. G. Baxter, Distillation Products, Inc., Rochester, New York.

Of a number of substances tested as protein precipitants in the preparation of the samples for analysis, only the zinc hydroxide achieved satisfactory precipitation. This technique yielded a protein-free supernatant fluid with recoveries of  $100 \pm 2$  per cent of nicotinamide which had been added to the original mixture. Negligible amounts of free color-producing substances were found in any of the dilute washed tissue preparations used in these experiments.

#### EXPERIMENTAL

Investigations showed that the optimum pH for enzyme activity is in the region of 7.2, this being the initial pH of the buffered homogenate (Fig 1). During the digestion period, no detectable shift in pH occurred. Preliminary tests were necessary, with each enzyme preparation, to deter-

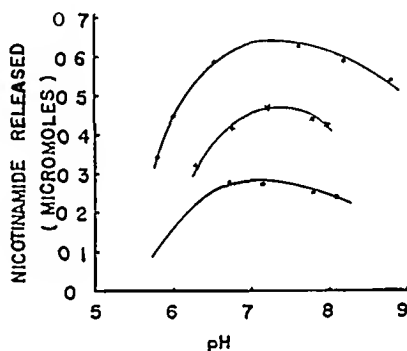


FIG 1 The effect of pH on the activity of DPN nucleosidase. The points represent the micromoles of nicotinamide released from 1.2 micromoles of DPN by three concentrations of the nucleosidase from brain on hydrolysis for 20 minutes at  $37^{\circ}$ .

mine the proper homogenate dilution which allowed approximately a 50 per cent hydrolysis of the substrate in 20 minutes. When the enzyme activity was low, some compromise was occasionally necessary to avoid difficulties in obtaining complete precipitation of protein from the digests.

It was found that the rate of hydrolysis of the added DPN was linear for at least 20 minutes under these conditions. Several determinations of the  $K_m$  of the nucleosidase gave values between  $1.0 \times 10^{-3}$  and  $5.6 \times 10^{-3}$  M within the range typical of hydrolytic enzymes. Since the enzyme preparations were by no means pure, no attempt was made to establish this value exactly.

A comparison of the activities of the various tissues studied is compiled in Table I from the results obtained on several groups of rats. Assays were run on pooled tissue samples. The data are not comparable to those

reported by Handler and Klein (2), for these workers found complete hydrolysis of DPN in the brain digests. It is quite probable, in the light of the present findings, that their preparation from brain tissue might have degraded much more DPN had higher concentrations been present.

In studying the inhibitory effect of  $\alpha$ -TPh on the nucleosidase, several difficulties were encountered. In unwashed preparations, inhibition by  $\alpha$ -TPh was not apparent, probably because of the presence of some impurity, soluble in saline, which inactivated or removed the  $\alpha$ -TPh. With washed preparations from brain there was a decided inhibition of nucleosidase activity with increasing amounts of  $\alpha$ -TPh. A typical result is shown in Fig. 2. Inhibition increased rapidly until the  $\alpha$ -TPh concentration reached 1.96 micromoles in each digest, further increases in inhibitor concentration caused a less rapid increase in the degree of inhibition. The data in Table II illustrate the effect of various levels of  $\alpha$ -TPh on the activity of the brain enzyme at several substrate concentrations.

TABLE I  
*Relative Activity of DPN Nucleosidase in Rat Tissue*

Tissue	Relative nucleosidase activity	Tissue	Relative nucleosidase activity
Brain*	100	Liver	22
Lung	28	Kidney	21
Heart	27	Muscle	8

\* The nucleosidase per gm. of dry brain, under the conditions of these experiments, split approximately 15 mμ of DPN in 20 minutes.

That the inhibition of nucleosidase activity by  $\alpha$ -TPh is also found with the crude enzyme prepared from lung, heart, muscle, kidney, and liver may be seen from the data in Table III. Quantitatively, much higher  $\alpha$ -TPh concentrations are required with tissues other than brain to achieve an equivalent degree of inhibition. This may be due to some interaction of the  $\alpha$ -TPh with protein which is present in larger amounts in the very concentrated homogenates of these other tissues.

Govier has demonstrated that digitoxin has an effect on succinodehydrogenase and on lactic dehydrogenase, which is similar to that of  $\alpha$ -TPh. The results shown in Fig. 3 tend to suggest that the digitoxin acts by a different mechanism, since no direct effect on the nucleosidase activity is evident.<sup>3</sup> It was necessary to include some alcohol in order to get appreciable amounts of digitoxin into solution. Control tests showed that any inhibition pro-

<sup>3</sup> A personal communication with Dr. Govier indicates that he has reached the same conclusion.

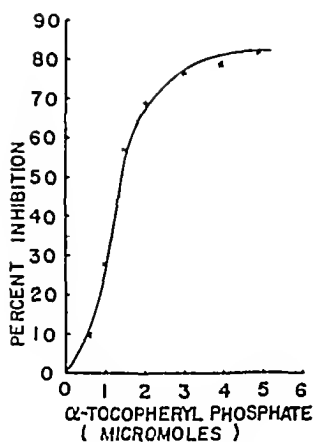


FIG 2

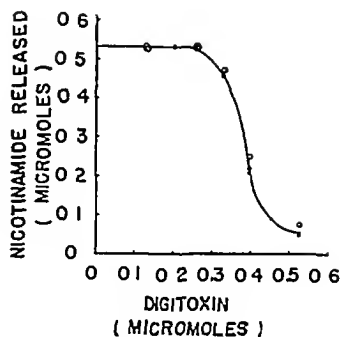


FIG 3

FIG 2 The inhibitory effect of  $\alpha$ -TPh on the DPN nucleosidase of rat brain homogenate. Each digest contained 1.2 micromoles of DPN.

FIG 3 The effect of digitoxin on the activity of DPN nucleosidase. Small amounts of alcohol were introduced to aid in the solution of the digitoxin. The eircled points indicate the effect of concentrations of alcohol alone which correspond to those amounts used in dissolving the glycoside.

TABLE II

*$\alpha$ -TPh Inhibition of Brain DPN Nucleosidase at Different Substrate Levels*

Micromoles in digest		Nicotinamide released	Per cent inhibition
DPN	$\alpha$ TPh		
		<i>micromoles</i>	
0.6	0	0.28	0
0.6	0.59	0.16	43
0.6	1.18	0.14	50
0.6	2.35	0.07	75
1.2	0	0.48	0
1.2	0.59	0.42	12
1.2	1.18	0.28	42
1.2	2.35	0.13	75
1.8	0	0.56	0
1.8	0.59	0.50	11
1.8	1.18	0.32	43
1.8	2.35	0.17	70

duced by the digitoxin in alcohol could be traced directly to the alcohol. This inhibition by alcohol seems to be due to precipitation of DPN rather than to a direct action on the nucleosidase.

Work done by Milhorat and Bartels (9), with patients suffering from muscular dystrophy, suggested that tocopherol forms a condensation product with inositol in the gastrointestinal tract. This inspired an in-

TABLE III

*Inhibition of DPN Nucleosidase of Various Tissues by  $\alpha$  TPh*

DPN concentration 1.2 micromoles in each digest

Nucleosidase source*	$\alpha$ TPh in digest	Nicotinamide released	Per cent inhibition	Nucleosidase source*	$\alpha$ TPh in digest	Nicotinamide released	Per cent inhibition
	<i>micromoles</i>	<i>micromoles</i>			<i>micromoles</i>	<i>micromoles</i>	
Brain	0	0.57	0	Liver	0	0.60	0
	1.9	0.10	82		1.9	0.60	0
Lung	0	0.51	0		9.8	0.53	12
	1.9	0.45	12	Kidney	0	0.38	0
	9.8	0.32	37		1.9	0.31	18
Heart	0	0.45	0	Muscle	0	0.32	0
	1.9	0.36	20		1.9	0.23	28
	9.8	0.19	58		9.8	0.19	41

\* Washed homogenate diluted to a suitable level

TABLE IV

*Effect of Inositol, with and without  $\alpha$  TPh, on DPN Nucleosidase of Rat Brain and Liver*

Enzyme source	Micromoles in digest		Nicotinamide released
	Inositol	$\alpha$ TPh	<i>micromoles</i>
Brain	0	0	0.37
	11.1	0	0.37
	22.2	0	0.37
	33.3	0	0.37
	0	1.96	0.09
	5.55	1.96	0.09
	11.1	1.96	0.08
	22.2	1.96	0.09
Liver	0	0	0.43
	0	8.69	0.17
	24.7	8.69	0.17
	24.7	0	0.49

vestigation of a possible effect of inositol on the nucleosidase system, either directly or through the  $\alpha$ -TPh inhibition mechanism. Table IV summarizes the results obtained. The inositol *in vitro* affected neither the activity of the nucleosidase nor the degree of its inhibition by  $\alpha$ -TPh. This was true



for both liver and brain homogenates, as is shown in Table IV. Incubating the inositol and liver homogenate, with and without  $\alpha$ -TPh, at 37° for 30 minutes prior to adding the substrate, did not affect the results shown in Table IV.

In 1942, Swingle *et al* (10) postulated that calcium ions accelerated the destruction of DPN by its nucleosidase. Since they followed the degradation by measuring residual DPN, there is no assurance that the enzyme stimulated by calcium ions is the nucleosidase. If such is indeed the case, this might be accomplished indirectly by removal of the  $\alpha$ -TPh inhibition of DPN nucleosidase. Such a mechanism has been proposed by Ames (11) who has presented evidence that calcium ions precipitate  $\alpha$ -TPh as the

TABLE V  
*Effect on Brain Nucleosidase of  $\text{Ca}^{++}$  in Presence and Absence of  $\alpha$ -TPh*

Micromoles in digest		Nicotinamide released
$\text{Ca}^{++}$	$\alpha$ TPh	
		<i>micromoles</i>
0	0	0.39
0	1.47	0.07
0.05	0	0.45
0.05	1.47	0.07
0.1	0	0.46
0.1	1.47	0.07
0.5	0	0.45
0.5	1.47	0.07
1.0	0	0.44
1.0	1.47	0.07
5.0	0	0.40
5.0	1.47	0.07

\*  $\text{CaCl}_2$

relatively insoluble monocalcium salt of TPh. He found that by addition of calcium chloride to the TPh-inhibited succinodehydrogenase system the inhibition was diminished and this he explained through the mechanism outlined by Govier *et al* (6). With the DPN nucleosidase, added calcium ions produced little or no change in the rate of substrate hydrolysis (Table V). When  $\alpha$ -TPh was added along with the calcium, no effect of calcium on the degree of enzyme inhibition by  $\alpha$ -TPh could be detected at molar ratios of  $\text{Ca} : \alpha$ -TPh from 1:29.4 to 1:0.29. A time-hydrolysis curve remained linear beyond the 20 minute digestion period usually employed, a stimulatory effect, therefore, was not masked by carrying the hydrolysis of the substrate too far. An attempt was made to duplicate the work of Swingle *et al*, except that the breakdown of added DPN was measured by estimating liberated nicotinamide. Contrary to their findings, no stimulation by

calcium ions was noted. The involvement of some other enzyme stimulated by calcium remains to be investigated.

### SUMMARY

1 The optimum pH for the activity of diphosphopyridine nucleotide nucleosidase has been established at about 7.2

2  $\alpha$ -Tocopheryl phosphate inhibits markedly the nucleosidase activity of washed homogenates of brain, liver, heart, kidney, lung, and muscle tissues of rats

3 No inhibition of the brain nucleosidase by digitoxin could be demonstrated

4 In the presence of inositol the ability of brain or liver nucleosidase preparations to hydrolyze DPN appeared to be unaffected. The inhibition of the nucleosidase by tocopheryl phosphate was not affected by the presence of inositol

5 Under the conditions of these experiments, the reputed activation of DPN nucleosidase by calcium ions could not be demonstrated, nor was there any indication of a reversal of the inhibition induced by tocopheryl phosphate when excess calcium ions were added to the system

6 The results of this investigation, in general, support the theory of Govier as an explanation of the mechanism by which tocopherol may inhibit the succinodehydrogenase system

The authors wish to express to Dr. A. M. Wynne, Department of Biochemistry, University of Toronto, their sincere appreciation for his helpful advice and criticism. Our thanks are also due to Dr. H. B. Speakman and Mr. W. C. Henry, Ontario Research Foundation, whose cooperation made this work possible.

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# MICROBIOLOGICAL DETERMINATION OF VALINE IN PROTEINS AND FOODS

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(Received for publication, July 14, 1947)

In contrast to the tedious, unreliable chemical methods, the microbiological assays for valine are easy and accurate. A wide selection of media and test organisms for the determination of this amino acid is offered in the literature (1-19).

Satisfactory standard curves were developed in this laboratory with both *Lactobacillus arabinosus* 17-5 and *Streptococcus faecalis* 9790. In assays reported here, *Streptococcus faecalis* was used with the medium reported for methionine (20) and modified as used for threonine (21).

## EXPERIMENTAL

*Streptococcus faecalis* 9790<sup>1</sup> was employed in the assays described.

**Basal Medium**—The basal medium was the same as that described in a previous paper for methionine (20), with the exception that pyridoxine was replaced by 400  $\gamma$  of pyridoxamine.

**Assay Procedure**—The procedures followed for the cultures, inoculum, and preparation of samples were identical with those described in other papers (20, 21).

**Preparation of Valine Standards**—Stokes and coworkers (5) have shown that the synthetic DL-racemate has exactly one-half the activity of the L isomer. Therefore, in preparing standard valine solutions and the standard curve, twice as much of the DL compound was used. The titration values on the standard curve (Fig. 1) were not altered by the addition to the medium of 1.2 mg. of any of the other nineteen amino acids.

Recovery of valine added to hydrolysates of arachin, glycemin, wheat germ, and whole wheat gave results well within the experimental error for this type of assay (Table I).

Table II shows values found for casein and several foods at different assay levels. Data on the reproducibility of values found for a number of materials when determined by separate assays are given in Table III.

<sup>1</sup> Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

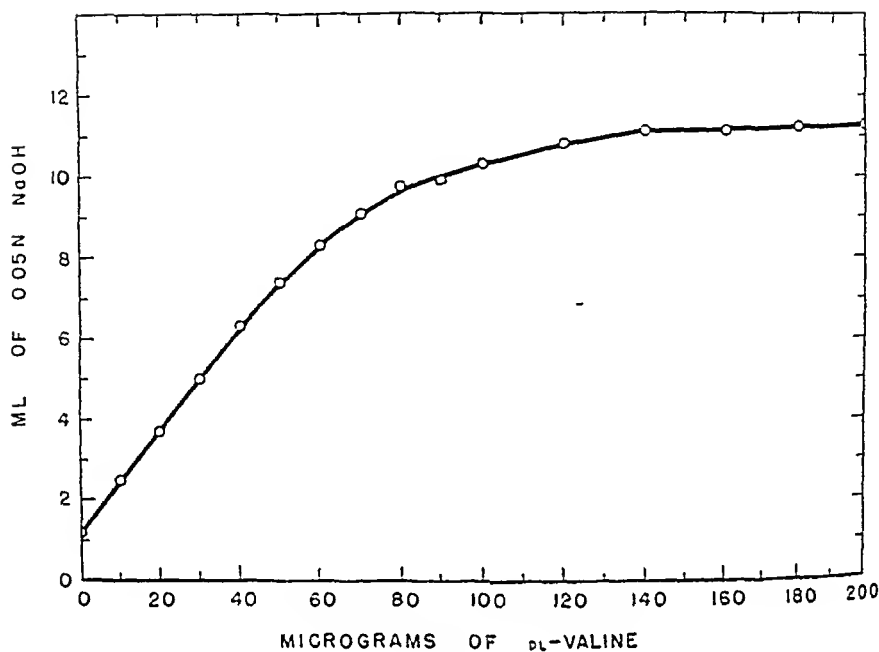


Fig 1

TABLE I  
Recovery of Valine Added to Protein Hydrolysates

Protein hydrolysate	Valine				
	In hydrolysate*	Added	Total	Found	Recovery
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent
Arachin	4.7	10.0	14.7	14.3	97
	9.4	10.0	19.4	19.3	99
Glycinin	14.1	10.0	24.1	24.8	103
	4.3	10.0	14.3	14.3	100
	4.3	20.0	24.3	24.8	102
	4.3	30.0	34.3	34.3	100
	4.3	40.0	44.3	43.8	99
Wheat germ	3.5	20.0	23.5	23.5	100
	7.0	20.0	27.0	26.8	99
	10.5	20.0	30.5	30.5	100
Wheat, whole	3.5	5.0	8.5	8.7	103
	3.5	10.0	13.5	13.5	100
	3.5	15.0	18.5	19.0	103
	3.5	20.0	23.5	24.2	103

\* Not corrected for moisture and ash

TABLE II  
*Valine Content at Different Assay Levels \**

Material	Level added	Found
Casein	$\gamma$	<i>per cent</i>
	100	6 30
	200	6 25
	300	6 50
	400	6 25
Average		6 33
Cottonseed flour	200	2 50
	400	2 55
	600	2 67
	800	2 50
Average		2 55
Ovalbumin	100	7 00
	200	7 00
	300	7 20
	400	7 20
Average		7 10
Oatmeal	500	0 84
	1000	0 85
	1500	0 83
	2000	0 83
Average		0 84
Rye, whole	1000	0 55
	2000	0 55
	3000	0 54
	4000	0 54
Average		0 55

\* Not corrected for moisture and ash

TABLE III  
*Reproducibility (Per Cent) of Valine Content When Determined by Separate Assays\**

Material	Assay 1	Assay 2	Average
Casein	6 49	6 33	6 41
Corn germ	1 15	1 11	1 13
Glycinin	4 30	4 26	4 28
Ovalbumin	7 07	7 10	7 08
Cottonseed globulin	5 32	5 40	5 36

\* Not corrected for moisture and ash



# THE TRYPTOPHAN CONTENT OF NORMAL HUMAN URINE

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(Received for publication, July 14, 1947)

Some time ago Albanese and Frankston reported urinary tryptophan outputs of 226 to 336 mg per day in seven normal men on normal diets (1). Such rapid wastage of a normal tissue constituent which is not synthesized at an appreciable rate, if it is at all, in the human organism (2) is hard to reconcile with present concepts of amino acid metabolism. A daily output of this much tryptophan exceeds the 0.2 gm estimated by Rose to be the minimal daily intake needed to maintain nitrogen equilibrium (3).

In a recent review, Albanese (4) reports that in the 24 hour urines of thirty normal human subjects the amino nitrogen of tryptophan accounted for an average of 4.8 per cent of the total 200 to 700 mg of free plus bound amino nitrogen. If this is true, the contribution of tryptophan to the total urinary amino nitrogen greatly exceeds its share in the total free plus bound amino nitrogen in dietary protein. In such an excellent dietary source of tryptophan as casein, the amino nitrogen present in tryptophan represents less than 2 per cent of the total  $\alpha$ -amino nitrogen that can be liberated by hydrolysis. The difference between a 2 per cent share in the  $\alpha$ -amino nitrogen intake and a 4.8 per cent share in the amino nitrogen output would seem to require the assumption either that considerable synthesis of tryptophan must occur in the human or that tryptophan is much more poorly metabolized than the average amino acid when fed in the form of protein.

Assays with *Lactobacillus arabinosus* have raised doubt concerning the conclusions of Albanese and Frankston by failing to confirm their observations. Schweigert, Sauberlich, and Elvehjem (5) could account microbiologically for the urinary excretion of only a tenth as much tryptophan per day (12.6 to 30.5 mg). Frankl and Dunn obtained closely similar results (12.8 to 22.9 mg) on 24 hour urines from normal adult male subjects on uncontrolled normal diets (6). In advancing possible reasons for the wide divergence between the results of Albanese and Frankston and their own, Schweigert, Sauberlich, and Elvehjem (5) suggested that certain tryptophan and indole derivatives measurable chemically may fail to induce a response in *Lactobacillus arabinosus*. This idea is echoed in a foot-note to the paper of Frankl and Dunn (6), which quotes Albanese as expressing the opinion that part of the tryptophan in the urine is bound, that both this fraction and the free tryptophan are measured by the Albanese and

Frankston method, but that *Lactobacillus arabinosus* responds only to free tryptophan

Careful study of the paper of Albanese and Frankston (1) seemed to indicate that their chemical procedure may have been inadequate. Their method of assay is essentially a modification of the Jolles color reaction for indican. Its performance was not compared with that of any other of the many well known chemical methods for estimating tryptophan. Its failure to account adequately for additions of tryptophan to the urine was ascribed arbitrarily to the solubility in the acid-urine mixtures of the mercuric sulfate complex precipitated to effect concentration of the tryptophan. Having had some experience with the method of Shaw and McFarlane (7), which is essentially a quantitative application of the well known Hopkins-Cole test, we became interested in determining whether this method would yield approximately the same or widely divergent results when applied to urines analyzed simultaneously with the Albanese-Frankston procedure. The purpose of this paper is to report our observations.

#### EXPERIMENTAL

Albanese and Frankston found their procedure insufficiently sensitive to warrant applying it without first concentrating the tryptophan in the urine by precipitation with mercuric sulfate. The sensitivity of the Shaw and McFarlane procedure is adequate, but interfering colors develop when it is applied directly to urine. Hence, the preliminary steps suggested by Albanese and Frankston, involving percolation of the urine through activated permutit to remove ammonia and other bases, extraction of the acidified filtrate with ether to remove indole and ether-soluble derivatives, precipitation with mercuric sulfate to concentrate the tryptophan, and solution of the precipitate in the trichloroacetic-hydrochloric acid reagent, were all used to obtain the solutions analyzed colorimetrically by the two methods.

*Preliminary Tests*—Albanese and Frankston noted that Beer's law did not apply strictly to the colors developed when their colorimetric procedure was used to measure various concentrations of pure tryptophan in water. This was confirmed by plotting readings made in a Klett-Summerson photoelectric colorimeter fitted with Filter S-54, and corrected for the reading of a reagent blank, against contents of tryptophan<sup>1</sup> ranging by intervals of 0.1 mg from 0.4 to 1.4 mg in the 2 ml of aqueous test solution. The colors produced ranged from a light green for low concentrations of tryptophan to a bright red for high concentrations. The calibration curve was similar to the one published by Albanese and Frankston (1).

<sup>1</sup> We are happy to acknowledge our indebtedness to Merck and Company, Inc., Rahway, New Jersey, for the supply of DL-tryptophan used in this study.



Calibration curves were also constructed from readings obtained by applying the Shaw-McFarlane procedure to 0.5 ml test samples containing tryptophan in amounts varying by 0.01 mg intervals from 0.01 to 0.14 mg. The readings were corrected for the color produced in a blank from which only the glyoxylic acid reagent was omitted. With either Filter S-52 or S-54, the results obtained fell on a straight line, indicating that in these cases Beer's law applied well.<sup>2</sup>

*Recoveries of Tryptophan from Urine*—Five aliquots of a mixed urine sample were analyzed in duplicate by both the Albanese-Frankston and the Shaw-McFarlane procedures. To the first aliquot no tryptophan was added, additions to the others amounted to 0.25 to 1.00 mg per 20 ml of sample from which the mercuric sulfate complex was precipitated. Results of the analyses are shown in Table I. The Albanese-Frankston procedure indicated the presence of much more tryptophan in the original

TABLE I  
*Tryptophan Recoveries from Urine by the Albanese-Frankston and Shaw-McFarlane Procedures*

Tryptophan added	Albanese Frankston procedure		Shaw McFarlane procedure	
		Completeness of recovery		Completeness of recovery
mg	mg	per cent	mg	per cent
0.0	1.7		0.25	
0.25	1.80	40.0	0.484	93.6
0.50	2.09	78.0	0.731	96.2
0.80	2.32	70.7	1.01	95.0
1.00	2.49	79.0	1.19	94.0

urine and accounted for considerably less of the added tryptophan than did the Shaw-McFarlane method.

Calculated per 100 ml of urine, the added tryptophan unaccounted for by the Albanese-Frankston procedure ranged from 0.55 to 1.05 mg and averaged 0.81 mg per 100 ml, in excellent agreement with the 0.5 to 1.05 mg and the average of 0.78 mg reported by Albanese and Frankston (1) as lost per 100 ml. Then assumption that the discrepancy was attributable chiefly to the solubility of the mercuric sulfate complex in the acid urine from which it was precipitated was not confirmed by assays with the Shaw-McFarlane technique. This method accounted for all but 0.10 to 0.30 mg of the added tryptophan per 100 ml. The average discrepancy

<sup>2</sup> Some grades of C P concentrated sulfuric acid produce a yellow colored blank. We have found Baker's C P special grade, low in nitrogen and arsenic, satisfactory. It is well also to use a good grade of copper sulfate.

was only 0.18 mg, 0.6 mg less per 100 ml than the loss reported by Albanese and Frankston. Analysis by the Shaw-McFarlane procedure of ten different aqueous solutions which contained 0.25 to 3.00 mg of tryptophan in the 20 ml precipitated with mercuric sulfate, but which were not first percolated through permutit, yielded smaller discrepancies. The largest divergence was 0.20 mg per 100 ml, the average 0.045 mg. If the poorer recoveries of tryptophan from the urine than from aqueous solution are attributable to the influence of the urine on the solubility of the mercuric sulfate complex, the effect is only a fifth to a sixth as great as suggested by Albanese and Frankston. It would be surprising indeed if absolutely no loss of tryptophan should occur in the passage of the urines through the column of permutit.

TABLE II  
*Apparent Tryptophan Content of 24 Hour Urine Samples*

Sample No	Albanese Frankston procedure	Shaw McFarlane procedure
	mg	mg
1a*	170.0	25.0
1b	173.0	31.2
1c	152.4	29.0
2a*	240.0	42.0
2b	164.8	29.6
2c	182.0	31.0
3	140.0	20.3
4	137.0	23.7
5	237.5	37.0
6	146.0	21.4
7	141.0	20.0

\* Three separate 24 hour samples were collected by Subjects 1 and 2

With the Shaw-McFarlane procedure it is possible to compensate for the color of the solution of the mercuric sulfate complex in the acid mixture by using a blank containing this solution and all of the reagents but the glyoxylic acid. Albanese and Frankston use only a reagent blank, which does not afford similar compensation. In our hands inclusion in the blank of the solution of the mercuric sulfate complex prepared from a normal urine and omission from it of the sodium nitrite and the acetic acid produced a color more intense than that obtained in a routine test of the same urine. We are therefore inclined to attribute the discrepancies in recoveries with the Albanese-Frankston procedure and the high estimates it gives of the tryptophan present in normal urine chiefly to errors inherent in the colorimetric technique.

*Analysis of 24 Hour Samples of Urine*—The Albanese-Frankston and Shaw-McFarlane procedures were finally applied simultaneously to eleven different 24 hour urine specimens collected from seven different male subjects ingesting average diets. The results are presented in Table II. The uncorrected outputs of 137 to 240 mg of tryptophan per day measured with the Albanese-Frankston colorimetric procedure were somewhat less than the values recorded by the originators of this method. The 20 to 42 mg of tryptophan indicated by analysis of the same urines with the Shaw-McFarlane colorimetric technique were correlated much more closely with the 12 to 30 mg per day obtained microbiologically by Schweigert, Sauberlich, and Elvehjem and the 12 to 22 mg noted by Frankl and Dunn than with the results obtained with the Albanese-Frankston procedure. The loss of tryptophan which may have resulted from the solubility of the mercuric sulfate complex or from the adsorption of tryptophan on the permutit could hardly have exceeded 6 mg per 24 hour sample.

#### SUMMARY

Comparisons of the Albanese and Frankston and the Shaw and McFarlane colorimetric procedures for estimating tryptophan indicate that the latter method is the more dependable and more accurate, as judged by variations in color development with concentration and capacity to measure accurately additions of tryptophan to urine. In 24 hour urine specimens, estimated by the Albanese and Frankston procedure to contain 137 to 240 mg of tryptophan, only 20 to 42 mg were measured by the Shaw and McFarlane procedure. The latter values are not markedly greater than the results with microbiological assays reported in the literature and they seem to conform more closely with the urinary excretion of tryptophan that might be anticipated from its indispensability and its low concentration in dietary proteins.

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# THE UTILIZATION OF D-AMINO ACIDS BY MAN\*

## VII PHENYLALANINE

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(Received for publication, May 17, 1947)

Existing data obtained from studies performed on humans and experimental animals indicate that two by-products (phenylpyruvic acid and L-tyrosine) result from the normal metabolism of naturally occurring phenylalanine in the mammalian organism. Evidence from the studies on alkaptonuria (1), tyrosinosis (2), phenylpyruvic oligophrenia (3), and vitamin C-deficient, premature infants (4) suggests that the amount of phenylalanine converted into these two substances and mono- and dihydroxy aromatic acids may vary according to the metabolic idiosyncrasies of the organism.

In addition to these differences in the mammalian metabolism of L-phenylalanine, considerable evidence of species differences in the utilization of D-phenylalanine is on hand. Feeding experiments with immature rats recently reported by Rose and Womack (5) reveal that the optical isomers of phenylalanine are almost equally effective in promoting growth when used to supplement a diet devoid of phenylalanine. In earlier studies Lewis and associates (6), however, found that rabbits excreted greater amounts of phenylpyruvic acid following subcutaneous or oral administration of D-phenylalanine than after the administration of L-phenylalanine. This observation and that of Levine and coworkers (7) on the infant indicated that D-phenylalanine is not utilized as readily as the L variety in the rabbit or the infant. In previously reported experiments (8), we found that the ingestion of racemic phenylalanine caused an increase in urinary phenylalanine in man equivalent to approximately 25 per cent of the D form occurring in the racemate. The failure of the administration of L-phenylalanine to raise appreciably the urinary levels of this amino acid led us to conclude that the D-phenylalanine is not completely utilized by man. Our subsequent studies on the metabolism of D-tyrosine indicated that measurements of the aliphatic urinary organic acids are required for a more complete evaluation of the utilization of the aromatic amino acids (9). Accordingly, we have repeated our investigations with the isomers of phenylalanine in order to ascertain the rôle of the organic acids in the

\* The work described in this report was supported by grants from the Rockefeller Foundation and the National Live Stock and Meat Board.

intermediary metabolism of phenylalanine. The results of these experiments disclose that great individual differences exist in the utilization of D-phenylalanine as measured by the excess urinary phenylalanine and organic acid output induced by the administration of the racemate, and that in some individuals far less D-phenylalanine is utilized than is indicated by the urinary phenylalanine measurement alone.

## EXPERIMENTAL

The DL-phenylalanine (Merck) employed in these studies was found to be optically inactive in water solution and to contain 8.30 per cent N

TABLE I

*Effect of Administration of 0.01 Mole of L- or DL-Phenylalanine on Output of Some Urinary Metabolites\**

Subjects	Phenylalanine			Tyrosine			Free phenols			Organic acids		
	Phenylalanine fed											
	None	L	DL	None	L	DL	None	L	DL	None	L	DL
	mg	mg	mg	mg	mg	mg	mg	mg	mg	cc 0.1 N HCl	cc 0.1 N HCl	cc 0.1 N HCl
A, ♂, 70 kilos	136	182	355	53.6	47.2	60.8	210	180	208	193	196	340
	125		472			69.0	162		204	161		320
			519									312
I, ♀, 60 "	145	158	475	56.9	66.5	63.4	248	216	228	197	249	317
			422			66.6			224			343
C, ♂, 6.0 " (infant)	84.6	120	362	28.2	32.0	36.3	123	127	102	191	215	230
L, ♀, 62 kilos	82	110	120	37.4	36.8	28.5	149	140	132	146	221	199
			192			43.2			184			183
			161			43.1			133			203

\* The values for adults represent the output for a 7 hour period following ingestion of the amino acid, those for the 6 month old infant are based on 24 hour collections

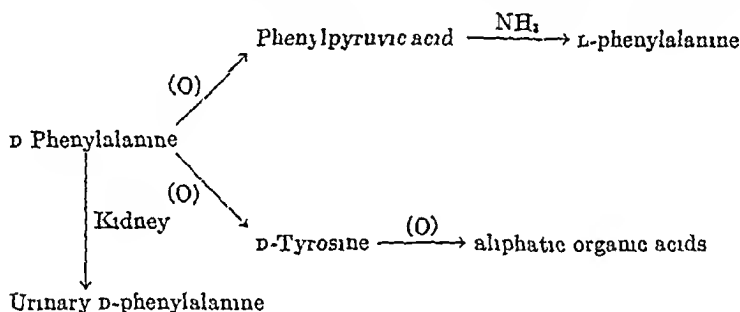
(theory, 8.48 per cent N) by micro-Kjeldahl analysis. The L-phenylalanine (Lemke) was found to have a specific rotation of  $[\alpha]_D^{25}$  (1.9624 gm per 100 cc of water) =  $-33.5^\circ$  (Fischer and Scholler (10),  $[\alpha]_D^{20} = -35.1^\circ$ ) and to contain 8.20 per cent N.

*Human Experiment*

After the elimination of the control urine specimens, which were collected 2 hours after breakfast, the subjects were given 0.01 mole (1.65 gm) of L- or DL-phenylalanine dissolved in 240 cc of water, 120 cc more water were also given at the end of each of the succeeding 6 hours to maintain uniform urine excretion. This period was selected on the basis of data which

showed that the phenylalanine output fell to normal after this interval had elapsed. The urines were pooled for the period of the experiment and then analyzed for total organic acids (11), ether-soluble free phenols (12), phenylalanine (8, 13), and tyrosine (9). Since the ingestion of food did not seem to affect the output of these metabolites, the normal food intake of the subjects was not restricted. In the study on the infant, the 0.01 mole of L- or DL-phenylalanine was given with the 10 a.m. feeding, and the urines collected during the succeeding 24 hours were analyzed for the constituents previously mentioned. All of the control experiments, in which no phenylalanine was fed, were similarly performed.

The analytical results obtained from these experiments are collected in Table I. It is to be noted that, although the excretion of phenylalanine and organic acids is increased in varying amounts in different subjects by the administration of DL-phenylalanine, the output of these substances is not raised above the control levels in any subject by the ingestion of



**L-phenylalanine** The non-aromatic nature of the excess of organic acids found in the urine of some of the subjects fed DL-phenylalanine is indicated by the negative tests for phenylpyruvic acid (14), hippuric acid (15), and benzoic acid (16) given by the samples. The similarity in output of ether-extractable phenols (Table I) subsequent to the administration of DL-phenylalanine to the output of these substances in the control and L-phenylalanine experiments also suggests that the excess organic acids resulting from the ingestion of the racemate are not hydroxy aromatic in character.

The production of these non-aromatic organic acids from the not easily fissionable benzene ring of phenylalanine is difficult to explain unless it is assumed that they arise from D-tyrosine which could be produced in the body by oxidation of the D component of the administered phenylalanine racemate. The scheme of the metabolic processes afforded by this speculation, which seems to fit the data presented in Table I, is shown in the accompanying diagram.

It also appears from our findings that the organism is not overwhelmed by the small doses of phenylalanine fed and that the metabolic intermediates of this scheme, phenylpyruvic acid and D-tyrosine, arising from the oxidation of D-phenylalanine are so rapidly converted to their respective terminal products as to avoid excretion in the urine. In the rabbit experiments of Lewis and his coworkers (6) and in the studies upon infants of

TABLE II

*Organic Acid and Phenylalanine Output for 7 Hour Period Following Administration of 0.01 Mole of L- or DL-Phenylalanine*

1.65 gm of DL phenylalanine = 0.83 gm of D phenylalanine

Subject	Normal output		Output after administration of phenylalanine isomers				Recovery in urine of D form fed	(b) - (a)		Tyrosine equivalent of organic acid excess†
			L (a)		DL (b)			Organic acids	I phenyl alanine	
	Organic acids*	Phenyl alanine	Organic acids	Phenyl alanine	Organic acids	I phenyl alanine				
	m eq HCl	mg	m eq HCl	mg	m eq HCl	mg		per cent	m eq HCl	
A, ♂, 70 kilos	19.8	136	19.6	182	31.0	355	21.0	+14.4	173	630
	16.1	125			32.6	472	35.0	+13.0	290	378
					31.2	519	40.6	+11.6	337	322
I, ♀, 60 "	19.7	145	24.9	158	31.7	475	38.2	+6.8	317	300
					34.2	422	31.8	+9.3	264	415
C, ♂, 60 "	19.1	146	21.5	120	23.5	362	29.2	+2.0	242	90
L, ♀, 62 kilos†	14.6	82	22.1	110	19.9	120	1.2	-2.2	10	0
					15.3	192	9.9	-0.8	82	0
					20.3	161	6.1	-1.8	51	0

\* The organic acid values were corrected for the phenylalanine content of the urine

† Mg of tyrosine = (milliequivalents of organic acid excess)/4 × 182 (mol wt of tyrosine)

‡ The negative organic acid increment of this subject indicates the over all errors involved in measurement of this kind and cannot, of course, be calculated to its tyrosine equivalent

Levine and associates (7) in which relatively larger doses of phenylalanine were employed, an excess of phenylpyruvic acid was excreted by the rabbit and an excess of both phenylpyruvic acid and tyrosine by the infant. In view of the fact that similar amounts of the amino acids were given to all the subjects, our results support the surmise that the production of the different metabolites represented by this scheme may not only be a function of dosage but also of the physiological characteristics of the individual.



Since the output of phenylalanine and the other metabolites reverts to the normal in 6 to 7 hours after the administration of racemic phenylalanine, the actual amount of urinary phenylalanine arising from the ingestion of the D variety can be approximated from the difference in phenylalanine output for the 7 hour period following administration of DL- and L-phenylalanine. The values so calculated are listed under the heading "(b) - (a)" in Table II and suggest that the feeding of 0.01 mole of DL-phenylalanine (equivalent to 0.005 mole of D-phenylalanine) produced an increment in urinary phenylalanine equivalent to 1.2 to 40.6 per cent of the D component. The quantity of D-tyrosine formed from the unnatural phenylalanine can be roughly estimated from the organic acid output if we assume that the molecular relationship of D-tyrosine to organic acids excreted (1.4), found

TABLE III

*Individual Variations in Utilization of D Phenylalanine by Man*  
1.65 gm. of DL-phenylalanine = 0.83 gm. of D-phenylalanine

Subject	Urinary phenylalanine determined colorimetrically	Phenylalanine equivalent of excess organic acids	Total D-phenylalanine metabolites recovered	D Phenylalanine not utilized
	mg	mg	mg	per cent
A, ♂, 70 kilos	173	595	768	92.5
	290	530	820	98.8
	337	480	817	98.4
I, ♀, 60 "	317	280	597	72.0
	264	380	544	65.6
C, ♂, 60 "	242	83	325	39.1
L, ♀, 62 "	10	0	10	1.2
	82	0	82	9.9
	51	0	51	6.1

in our previous report (9), applies in this instance also. The results of calculations based on these premises are shown in Table II and demonstrate that the tyrosine equivalent of the organic acid excess, like the phenylalanine output, varies with each subject tested. The failure of the administration of DL-phenylalanine to cause a rise in phenylalanine, organic acids, and tyrosine or other aromatic acids in Subject L, indicates that some individuals are capable of utilizing D-phenylalanine more completely than others. Subject A represents the opposite extreme. The metabolic divergence of these two individuals with respect to D-phenylalanine is of interest in view of their failure to exhibit such a diversity with respect to the metabolism of D-tyrosine (9).

Inasmuch as the organic acid attributed to D-tyrosine probably arises from the oxidation of an equivalent amount of D-phenylalanine (see the

diagram), the total amount of D-phenylalanine not utilized can be ascertained from the sum of the organic acid excess computed as phenylalanine and the urinary phenylalanine found colorimetrically. The results of these computations are listed in Table III and serve to emphasize further the metabolic individuality of the subjects.

### *Comments*

The data presented in this report and evidence previously reported by others indicate that the processes involved in the human metabolism of both L- and D-phenylalanine are susceptible to greater modifications than appear to occur for the other amino acids. Indeed, the variability exhibited with respect to the utilization of D-phenylalanine greatly exceeds that found with the D form of other amino acids so far tested by us. The immediate factors responsible for these variants seem to arise from differences in rates of oxidation of phenylalanine, of either optical variety, to tyrosine and phenylpyruvic acid. Although some of the variations arising from differentials in the oxidations of L-phenylalanine may be related to differences in available vitamin C (17), it does not appear likely from the recent report of Basinski and Sealock (18) that the diversity in the metabolic handling of D-phenylalanine can be attributed properly to the same cause. Consequently, in the absence of experimental evidence other than the excretion data, we are led to suspect that the observed individual differences in the metabolism of D-phenylalanine are probably due to qualitative or quantitative variations in the D-amino acid oxidase content of the tissues of different subjects. The evidences and implications of biochemical individuality in man have recently been discussed by Williams (19). The often observed nutritional differences of different strains of the rat, mouse, and other laboratory animals may also be related in part to variations in their enzyme components.

### SUMMARY

A reinvestigation of the metabolism of D-phenylalanine in man in terms of quantitative differences in metabolites occurring in the urine in the 7 hours following the administration of L- and DL-phenylalanine suggests that, whereas D-phenylalanine may be readily utilized by some individuals, in others the major portion of the ingested D-phenylalanine may be oxidized to D-tyrosine, which subsequently undergoes oxidative fission and is lost to the organism in the form of urinary aliphatic organic acids. The possible relationship of these findings to the known aberrations in the metabolism of the aromatic amino acids in man is discussed.

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# FACTORS AFFECTING FOLIC ACID DETERMINATION

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(Received for publication, July 8, 1947)

In the preparation of sample solutions used in the microbiological assay for folic acid various extraction methods have been employed. The frequent occurrence of folic acid as complex conjugates which must be hydrolyzed to pteroylglutamic acid for maximum utilization by microorganisms necessitates use of enzyme or chemical hydrolysis in the extraction procedure. The question of stability of folic acid under such treatment, as well as under conditions of sterilization and storage involved in microbiological procedures, has been of concern in this laboratory. On the other hand we have studied means of removing folic acid from its natural sources in attempts to prepare folic acid-free growth-promoting supplements suitable for use in basal media. The destructive effects of heat, light, and bisulfite have been measured, and methods of removing folic acid from solution by adsorbing agents have been studied. The purpose of this report is to summarize briefly the results of these experiments which have added to our knowledge of the properties of folic acid.

## EXPERIMENTAL

The observations outlined here were made on crystalline folic acid, tomato juice serum (canned tomatoes filtered through sintered glass), and two samples of dehydrated liver extract powder, one a concentrate high in folic acid,<sup>1</sup> the other an antipernicious anemia preparation.

It will be noted that these are soluble materials which can be added directly to the assay medium without preliminary extraction, thereby permitting through comparison of treated and untreated portions an evaluation of the effects of various treatments on the folic acid present.

Such a comparison is difficult in the study of such materials as cereal products, animal tissues, and rat feces, which require various types of manipulation before the sample can be introduced into the medium for microbiological assay.

In the quantitative estimation of folic acid, *Lactobacillus casei* was used with variously proposed media (1-3) and modifications thereof. The standard folic acid solution serving as a reference standard was prepared

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<sup>1</sup> This preparation was kindly furnished by Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company.

from a stock solution containing 100  $\gamma$  per ml of the crystalline vitamin in 0.01 N NaOH in 20 per cent EtOH. Standard solutions for assay purposes were freshly prepared for each series of folic acid tests by diluting this stock solution with distilled water to a concentration of 0.0002  $\gamma$  per

TABLE I  
*Stability of Folic Acid under Different Conditions*

Substance	Concentration	Treatment*	Loss of folic acid
	$\gamma$ per ml		per cent
Crystalline folic acid	100	Standard, 0.01 N NaOH in 20% EtOH, stored in refrigerator at 1°, 3 mos	0
	0.0002	Diluted standard in H <sub>2</sub> O, filtered†	0
	10	Autoclaved, 121°, 30 min	100
		pH 1	40-45
		" 3	10-30
Liver extract preparation, high in folic acid		" 1-12	<0
	1	Autoclaved, 121°, 30 min, basal medium, pH 6.8	75-80
		Sulfite, room temperature, 24 hrs, pH 5	
	per cent		
	0.5	Autoclaved, 121°, 15 min, 0.05 N NaOH	0
		Autoclaved 121°, 30 min	70-80
		pH 1	35-40
		" 3	10-20
		" 1-12	90
	1	Autoclaved, 121°, 90 min, pH 1	†
		" 121°, 30 " 0.2 N and 1.0 N HCl	§
		Heated in oven, 60°, 1 hr, pH 1	
		" " " 60°, 1 " " 3	¶
		" on hot-plate, 100°, 15 min, pH 3	
		Sulfite, room temperature, 24 hrs, pH 5	50-60
		Sulfite, autoclaved at 121°, 15 min, pH 5	20-25
		Irradiated, 500 watt Mazda lamp, 6 hrs, pH 1, 100 ml over 72 sq in, 1 ft distance	60
		Same, pH 5.5	<0
	2	Autoclaved, 121°, 15 min, 0.05 N NaOH	0

TABLE I—*Concluded*

Substance	Concentration	Treatment*	Loss of folic acid
	<i>per cent</i>		<i>per cent</i>
Liver extract, anti-pernicious anemia preparation	0.5	Autoclaved, 121°, 30 min	
		pH 1	70
		" 3	40
		" 4	20
		" 5-7	10
		" 9	40 (Gain)
		" 10-12	15 (Gain)
	1	Autoclaved, 121°, 90 min, pH 1	80-100
		" 121°, 90 "	25-35
		pH 5.5	
		Heated, 100°, 1 hr in O <sub>2</sub> , pH 10	50-60
Tomato juice serum, filtered canned tomatoes	Undiluted	Sulfite, room temperature, 24 hrs, pH 5	30-40
		Irradiated, Hanovia lamp, 3 hrs, pH 4.3, 100 ml over 72 sq in, 1 ft distance	0
		Same, 7 hrs	0

\* Unless otherwise indicated, aqueous solutions adjusted to the desired pH by means of NaOH or HCl were used

† Schleicher and Schull No. 589 filter paper

‡ Under these conditions the loss of folic acid was 5 times greater at 1 N HCl than at 0.2 N HCl

§ Less folic acid was lost in this case than in autoclaving at 121° for 30 minutes at pH 3

|| The loss of folic acid was less than under conditions of heating in an oven at 60° for 1 hour at pH 1

¶ This treatment produced a loss equivalent to that resulting from heating in an oven at pH 3 for 1 hour at 60°

ml The stability of this stock solution under the usual conditions of storage in a refrigerator at 4° was determined

Since it is frequently necessary in certain extraction procedures to employ filtration, it was desirable to ascertain whether loss of vitamin occurred as a result of adsorption on the filter paper. The suitability of Schleicher and Schull No. 589 paper was determined

Experiments were carried out in which folic acid in concentrations of 1  $\gamma$  per ml was autoclaved in the *Lactobacillus casei* basal medium, at pH 6.8, for a period of 30 minutes, thereby allowing a margin of 15 minutes beyond the time recommended for sterilization in the microbiological assay

Dual interest, first in the quantitative extraction of folic acid without

loss, and second in the complete destruction of the vitamin, prompted the study of the effect of heat over a wide range of pH. Chemical and irradiation treatments were made in an effort to effect complete destruction. The pH of all samples was adjusted in aqueous solutions by the addition of either HCl or NaOH. Sulfite treatment involved the addition of  $\text{Na}_2\text{SO}_3$  and HCl in molecular proportion to produce  $\text{NaHSO}_3$ , according to the procedure

TABLE II

*Adsorption of Folic acid (Crystalline)\* from 0.1 N NaOH and 0.004 N HCl Solutions Estimated Visually by Relative Fluorescence*

Adsorbing agents	Degree of adsorption†	
	0.1 N NaOH solution	0.004 N HCl solution
Super Filtrol (thiamine grade)	+	+++
Activated Decalco (for thiamine determination), 2 different samples	+	+
Activated alumina, 3 different sources	+	+
MgO	+	++
Adsorptive powdered magnesia, No. 2641, Westvaco	++++	++++
MgO, 1 part } Al <sub>2</sub> O <sub>3</sub> , 1 part } Celite, 1 part }	++	++
Bentonite	+++	++
Fullers' earth	+	+++
Lloyd's reagent	+	++
Amberlite IR-1	+	+
" IR-100	++	+
" IR-4	+	+
Symacar	++	++
Carboraffin	++++	++++
CRBX "E"	+++	++++
Norit A	++++	++++
Dareo G-60	++++	+++

\* 25  $\gamma$  per ml

† The plus signs have been evaluated qualitatively as follows: + poor, ++ fair, +++ good, ++++ very good

routinely employed for thiamine destruction (4). Oxygen, directly from a tank, was bubbled for 1 hour through a 1 per cent solution of the anti-pernicious anemia liver extract adjusted to pH 10 and held at 100°. Irradiation for a period of 6 hours was carried out by means of a 500 watt Mazda lamp at a distance of 1 foot on a 2 per cent solution of the liver extract preparation high in folic acid. 100 ml samples adjusted to pH 1 and 5.5 respectively and spread over a surface of 72 sq in. were used. Samples



of undiluted tomato juice serum having a pH of 4.3 were irradiated for periods of 3 and 7 hours respectively with a Hanovia lamp of the high pressure mercury arc type. In this case also, irradiation was effected on 100 ml

TABLE III

*Estimated Adsorption of Folic Acid from Liver Extract Solution and Tomato Juice Serum Following One to Three 1 Hour Treatments with Specified Adsorbents*

Treatment	Degree of adsorption
Liver extract, antipernicious anemia preparation, 2.5% solution in 0.004 N HCl + 100 mg adsorbing agent per 25 ml solution	
Darco G-60, 1 adsorption	Small
" " 3 adsorptions	Great
Norit A, 1 adsorption	Negligible
" " 3 adsorptions	Less than half
CRBX "E", 1 adsorption	Small
" " 2 adsorptions	Incomplete
" " 3 "	Great
Carboraffin, 1 adsorption	Small
" " 2 adsorptions	Incomplete
" " 3 "	Great
Fuller's earth, 1 adsorption	Negligible
" " 3 adsorptions	"
Magnesia No 2641, 1 adsorption	"
" " 2641, 2 adsorptions	"
" " 2641, 3 "	"
Super Filtrol, 1 adsorption	Very small
" " 3 adsorptions	Slight
Tomato juice serum, undiluted, 5 mg adsorbing agent per ml serum in each case	
Magnesia No 2641, 3 adsorptions, pH 4.3	None
Darco G-60, 1 adsorption, pH 2.68	Incomplete
" " 2 adsorptions " 2.68	Complete
" " 1 adsorption " 4.3	Almost none
" " 2 adsorptions " 4.3	Incomplete
" " 3 " " 4.3	Complete
" " 1 adsorption " 9.1	Almost none
" " 2 adsorptions " 9.1	Incomplete, < pH 4.3
" " 3 " " 9.1	Complete

samples spread over an area of 72 sq. in. at a distance of 1 foot from the lamp. The data for all of these experiments are shown in Table I.

In Table II are outlined the results of qualitative experiments designed to determine the relative effectiveness of various adsorbing agents in re-

moving folic acid from solutions containing 25  $\gamma$  per ml of the crystalline vitamin in 0.1 N NaOH and 0.004 N HCl respectively. Becker and Schöpf (5) have recommended the adsorption of pterins from 0.004 N HCl solutions. The brilliant blue fluorescence of folic acid in aqueous solutions when exposed to a source of ultraviolet light afforded a criterion for determining the degree of adsorption of this compound by each of the substances tested. Tubes containing 5 ml of a solution of crystalline folic acid (2  $\gamma$  per ml) in 0.1 N NaOH and 0.004 N HCl respectively were used as standards and by visual comparison the degree of adsorption in these preliminary experiments was estimated as very good, good, fair, and poor.

Based upon the findings of these qualitative tests, seven of the most effective adsorbing agents for folic acid were selected for use on the antiper-nicious anemia liver extract sample. In addition, one of the activated carbons, Darco G-60, and magnesia No. 2641 were used at three different pH levels of the tomato juice serum. The degree of adsorption of folic acid was determined microbiologically upon the filtrate by comparison of the growth of *Lactobacillus casei* in tubes containing basal medium plus 1 ml of treated tomato juice serum or 1 ml of the adsorbed liver extract solution (equivalent to 5 mg of liver extract) with that obtained with the same quantities of the respective untreated samples. Negative controls (basal medium without sample or folic acid) as well as routine controls (medium to which folic acid was added in amounts sufficient to produce a maximum effect) served as reference points for this series. The results of these experiments are given in Table III.

#### RESULTS AND DISCUSSION

The stock folic acid solution used as a reference standard was found to be stable after storage for 3 months at 4°. No loss was found upon filtering folic acid solutions through Schleicher and Schull No. 589 filter paper, which is suitable also in riboflavin extraction procedures.

In efforts to prepare extracts free from folic acid, it was found that sulfite treatment destroyed more than half of that present, but in no instance was destruction complete. This procedure is therefore of little value in the preparation of growth-promoting supplements free from folic acid.

The use of irradiation with either the Hanovia or the Mazda lamp was also found to be an ineffective method for destroying folic acid.

Heating at pH 10 in a stream of oxygen destroyed the folic acid present in liver extract to the extent of 50 to 60 per cent. This procedure is consequently of little practical value in obtaining desirable supplements.

In general, loss of folic acid is greater in the case of crystalline vitamin in unbuffered solutions than in natural sources of this substance. At pH 3 folic acid is readily destroyed under most conditions and as the pH is

lowered it becomes progressively more unstable. Autoclaving for a period as short as 30 minutes at 121° and pH 1 causes 70 to 100 per cent destruction. In the range from pH 4 to 12, while the destruction is slight in comparison with that in the lower ranges, the loss is nevertheless significant. No loss was found upon autoclaving in the basal medium (pH 6.8) for 30 minutes. Thus sterilization in the microbiological assay may be carried out without loss of the vitamin.

A clear understanding of the stability of folic acid in the pernicious anemia liver extract is obscured by other factors. With increasing pH, above 7.0 the apparent folic acid increased during autoclaving at 121° for 30 minutes over that in the untreated sample to a maximum gain of about 10 per cent at pH 9, when the activity began to decrease with further increasing pH. At pH 12 it was still significantly greater than in the untreated extract. It would appear then that under these conditions alkali either frees bound folic acid in the liver extract, making it available to *Lactobacillus casei*, or liberates growth-stimulating substances not available in the untreated sample and not present in the basal medium.

These findings bring forcibly to attention the question of suitability of procedures for folic acid extraction. Data relative to the quantitative extraction of this vitamin for purposes of analysis have not been presented here, since there are still far too many unanswered questions to permit any satisfactory recommendation in cases of more complex materials. In many instances a preliminary hydrolysis of the substance is necessary to free the folic acid, followed by enzyme treatment for the purpose of releasing those bound forms not otherwise available to the organism. For example, one sample of yeast extract subjected to 7½ hours of autoclaving at pH 4 yielded a folic acid value in a slightly modified Teply medium (2) with *Streptococcus faecalis* R 10 times that found for the untreated sample. Subsequent enzyme treatment resulted in a value greater by 4 times that from autoclaving alone. Data are lacking relative to the completeness of extraction or degree of accompanying destruction in this case, as well as in work reported in the literature. Maximum values often used as a criterion are insufficient proof of true vitamin potency.

Of a number of adsorbing agents that have been found useful in the study of organic compounds, only five were found to have any important capacity for the adsorption of folic acid under the conditions employed.

The use of fluorescence offered a rapid and convenient means for estimating the degree of adsorption from solutions of the crystalline vitamin. It is suggested that the use of fluorescence may prove of value in the quantitative estimation of folic acid upon the selection of proper filters. A study of the use of this technique is being continued.

The results reported in Table II permitted a selection of the adsorbing

agents that showed most promise and these were used subsequently with the more complex solutions of liver extract and tomato juice serum. These latter studies, presented in Table III, indicate that the activated charcoals, Darco G-60, CRBX "E", and carboraffin, were most effective in removing folic acid.

When the pH was varied and Darco G-60 was used, it was found that, of the three conditions studied, adsorption was greatest at the lowest pH (2.68). Under these conditions folic acid was completely removed from tomato juice serum with two adsorptions. Magnesia No. 2641 was completely ineffective in its adsorbing capacity, three adsorptions at pH 4.3 being required, as contrasted with complete adsorption by Darco G 60 with the same number of treatments at the same pH.

#### SUMMARY

The stability of folic acid under conditions of extraction, sterilization, and storage involved in microbiological assay procedures for this vitamin has been studied. No loss of folic acid was observed during the sterilization process, or during 3 months storage of stock solutions. Destruction during autoclaving for 30 minutes at pH 3.0 is marked, however, and becomes progressively greater with lowered pH.

In the interest of preparing folic acid-free supplements suitable for addition to the basal medium, methods of removing folic acid from natural sources such as liver extract and tomato juice serum were examined. The use of heat in acid solution, irradiation, or treatment with sulfite was unsatisfactory for this purpose. Of a number of adsorbing agents studied activated charcoals were most efficient in removing folic acid from tomato juice serum, and a pH below 3.0 was most suitable for this purpose. For aqueous solutions of folic acid visual comparison of intensity of fluorescence after adsorption treatment was used to evaluate the adsorbing agents studied.

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# GROWTH INHIBITION OF BACTERIA BY SYNTHETIC PTERINS

## II STUDIES WITH *ESCHERICHIA COLI*, *STAPHYLOCOCCUS AUREUS*, AND *LACTOBACILLUS ARABINOSUS* SHOWING SYNERGISM BETWEEN PTERIN AND SULFONAMIDE\*

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(Received for publication, July 11, 1947)

Daniel, Norris, Scott, and Heuser (1) reported that several 2,4-diamino pterins inhibit the growth of *Streptococcus faecalis* and *Lactobacillus casei*, which require preformed folic acid, and *Lactobacillus arabinosus*, which synthesizes its own needs of the vitamin. Lampen and Jones (2) have shown that sulfonamides inhibit growth of bacteria by interfering with the synthesis of folic acid. The inhibitory pterins are believed to stop bacterial growth by preventing the use of folic acid.

The studies reported here demonstrate that several of these pterins also inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*, which synthesize folic acid, and show that a synergistic effect is obtained by adding both pterin and sulfonamide to the basal media of these bacteria and *Lactobacillus arabinosus*.

### EXPERIMENTAL

*Cultures and Media*—The cultures of *Escherichia coli* and *Staphylococcus aureus* were obtained from Dr. I. C. Gunsalus, Department of Bacteriology, Cornell University, and the culture of *Lactobacillus arabinosus* from the American Type Culture Collection. The culturing of the latter organism and the medium employed for assay have been described in the previous report (1). *Escherichia coli* and *Staphylococcus aureus* were carried on agar slants composed of 0.3 per cent beef extract, 0.5 per cent peptone, and 1.5 per cent agar. Weekly transfers were made through broth of the same composition with the omission of agar.

\* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D. C., and was aided by grants to Cornell University by the Nutrition Foundation, Inc., New York, and the Western Condensing Company, Appleton, Wisconsin. The work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

The medium used for *Escherichia coli* contained 5.0 gm of  $\text{NH}_4$  citrate, 3.0 gm of  $\text{KH}_2\text{PO}_4$ , 3.0 gm of  $\text{NaCl}$ , 0.2 gm of  $\text{Na}_2\text{SO}_4$ , 0.05 gm of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 15.0 mg of niacin, 2.0 gm of glucose, 0.5 gm of casein hydrolysate, and 30 ml of single strength *Streptococcus faecalis* medium (3) made up to 1 liter with  $\text{H}_2\text{O}$  at pH 6.5 ml of this medium were placed in each assay tube and the supplements were added to a total volume of 10 ml.

For *Staphylococcus aureus* the medium of Kligler, Grossowicz, and Bergner (4) was used. This was modified to contain  $\text{NaCl}$  10 gm,  $\text{Na}_2\text{HPO}_4$  7 gm, casein hydrolysate 10 gm, L-tryptophan 200 mg, glucose 4.5 gm, thiamine 4  $\gamma$ , niacin 400  $\gamma$ , and water to a volume of 1 liter at pH 7.2 to 7.4. Supplements were added to 5 ml of this medium and the final volume made to 10 ml. 50 ml Erlenmeyer flasks were employed for assay vessels instead of culture tubes, because of the greater surface afforded for contact with air.

The culture of *Escherichia coli* did not grow well on the inorganic salts-glucose medium, but grew very rapidly in a complete medium described by Snell, Guiard, and Williams (3). The organism was adapted to the salts-glucose medium over a period of several weeks by gradually decreasing the proportion of complete medium and increasing the simple medium, as reported by Shive and Macow (5). *Escherichia coli* was then carried by daily transfers into 10 ml of salts-glucose medium supplemented with 0.15 ml of complete medium. After 20 to 24 hours incubation at  $37^\circ$ , the culture was used to inoculate the assay tubes by transferring a loopful of inoculum into each tube. The tubes were incubated at  $37^\circ$  for 16 to 18 hours. After 4 weeks of this daily transfer, the culture gradually became less active, and readaptation was necessary.

*Staphylococcus aureus* was transferred from the stock slant to a nutrient agar slant and grown for 20 to 24 hours at  $37^\circ$ . The organisms were scraped off by sterile loop and suspended in 10 ml of sterile saline, until the turbidity of the suspension read 20 to 30 on the Coleman spectrophotometer when set at a wave-length of  $650\text{ m}\mu$ . The assay flasks were inoculated by loop and incubated at  $37^\circ$  for 16 to 18 hours. At the end of the incubation period, the growth of all organisms was measured turbidimetrically by use of a Coleman spectrophotometer.

*Compounds Used*—The synthetic pterins<sup>1</sup> used in these studies have been described previously (1). The names of these compounds are presented in Table I.

*Methods*—In studies with *Escherichia coli* and *Staphylococcus aureus* increasing amounts of pterin and sulfathiazole were added alone and in combination to the assay vessels. With *Lactobacillus arabinosus* the

<sup>1</sup> The synthesis of these compounds is reported by Mallette, M. F., Taylor, E. C., Jr., and Cain, C. K. (*J. Am. Chem. Soc.*, 69, 1814 (1947)).

TABLE I  
*Synthetic Pterins Studied*

Compound No	Name
1	2,4-Diamino 6,7 dimethylpyrimido(1,5-b)pyrimine
2	2,4-Diamino 6,7 dicarboxypyrimido(1,5-b)pyrimine
3	2,4-Diamino 7 carboxypyrimido(1,5-b)pyrimine
4	2,4-Diamino 6,7 diphenylpyrimido(1,5-b)pyrimine
5	2,4-Diaminopyrimido(1,5-b)pyrimine

TABLE II  
*Inhibition of Growth of Escherichia coli by Synthetic Pterins and Sulfonamides*

Compound No	Compound added	Sulfonamide added*	Galanometer reading†	Compound No	Compound added	Sulfonamide added	Galanometer reading
	$\gamma$ per 10 ml	$\gamma$ per 10 ml			$\gamma$ per 10 ml	$\gamma$ per 10 ml	
1	0	0	36	1	300	0	38
	0	5	38		600	0	61
	0	10	39		900	0	76
	0	20	52		1200	0	70
	0	50	86		1500	0	84
	0	100	91		100	0	36
	1000	0	66		100	5	18
	2000	0	88		100	10	59
	3000	0	95		100	20	96
	100	0	36	5	1000	0	36
2	100	5	42		2000	0	16
	100	10	57		3000	0	52
	100	20	88		1000	0	61
	1000	0	36		5000	0	69
	5000	0	47		1000	5	11
	1000	5	40		1000	10	46
	1000	10	50		1000	20	75
	1000	20	77	3	1000	0	37
	1000	0	37		5000	0	56
	5000	0	56		1000	5	38
	1000	5	38		1000	10	39
	1000	10	39		1000	20	52

\* Sulfathiazole

† A reading of 100 represents no growth

synergistic effect was shown by adding graded levels of sulfathiazole to an ineffective level of pterin. The antagonistic effect of folic acid<sup>2</sup> on the

<sup>2</sup> The authors are indebted to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for synthetic folic acid.

pterin and sulfonamide inhibition was shown for each organism by adding varying amounts of the vitamin

### Results

*Synergism of Pterin and Sulfonamide*—The results of studies with *Escherichia coli* and *Staphylococcus aureus* are presented in Tables II and III. When added alone, the dimethyl (Compound 1) and diphenyl

TABLE III  
Inhibition of Growth of *Staphylococcus aureus* by Synthetic Pterins and Sulfonamide

Compound No	Compound added	Sulfonamide added*	Galvanometer reading†	Compound No	Compound added	Sulfonamide added	Galvanometer reading
	$\gamma$ per 10 ml	$\gamma$ per 10 ml			$\gamma$ per 10 ml	$\gamma$ per 10 ml	
1	0	0	15	3	1000	0	25
	0	5	22		5000	0	24
	0	10	24		1000	5	78
	0	20	30		1000	10	88
	0	50	18		1000	20	91
	0	100	68		300	0	49
	0	400	82		600	0	64
	0	1000	85		900	0	72
	500	0	14	5	1200	0	86
	1000	0	74		1500	0	90
	1500	0	96		20	0	16
	20	0	13		20	5	27
	20	5	16		20	10	62
	20	10	28		20	20	71
	20	20	91		1000	0	24
	20	20	91		5000	0	39
2	1000	0	19		1000	5	37
	5000	0	17		1000	10	61
	500	0	14		1000	20	92
	500	5	19				
	500	10	79				
	500	20	95				

\* Sulfathiazole

† A reading of 100 represents no growth

(Compound 4) pterins were the most effective in inhibiting the growth of both organisms, although *S. aureus* seemed to be more susceptible to pterin inhibition than *E. coli*. The combined addition of a low level of pterin and sulfonamide produced a marked inhibition of the growth of *E. coli* and *S. aureus*, showing considerable synergism between these two compounds. The carboxy pterins (Compounds 2 and 3) had little effect on the growth of *E. coli* with or without sulfathiazole, whereas with *S. aureus* these acid derivatives showed a definite synergistic effect when



added with the sulfonamide Compound 5, 2,4-diaminopyrimido(4,5-b)-pyrazine, exhibited only slight antibacterial action on *E coli* and *S aureus*,

TABLE IV

*Inhibition of Growth of Lactobacillus arabinosus by Synthetic Pterins and Sulfonamide*

Compound No	Compound added	Sulfonamide added*	Galvanometer reading†	
			Without PABA	With PABA‡
	$\gamma$ per 10 ml	$\gamma$ per 10 ml		
	0	0	10	5
	0	10	33	13
	0	50	39	20
	0	100	45	48
	0	1,000	55	56
	0	10,000	60	65
1	100	0	40	5
	100	10	66	64
	100	100	79	75
	100	1,000	90	82
2	1000	0	10	5
	1000	10	59	20
	1000	100	58	49
	1000	1,000	58	64
3	1000	0	11	5
	1000	10	60	52
	1000	100	75	70
	1000	1,000	85	85
4	2	0		6
	2	10		44
	2	100		78
	2	1,000		86
	0.5	0	37	
	0.5	10	71	
	0.5	100	78	
	0.5	1,000	82	
5	1000	0	28	5
	1000	10	63	25
	1000	100	69	61
	1000	1,000	67	75

\* Sulfathiazole

† A reading of 100 represents no growth

‡ 1  $\gamma$  of PABA added per tube

but when added with sulfathiazole showed a considerable synergistic effect, especially with *S aureus*

The results obtained with *Lactobacillus arabinosus* are presented in Table IV. Studies were made with and without the addition of *p*-amino-

benzoic acid (PABA) to the medium. The amount of PABA present in the medium was so small that it could not be detected by the usual *Acetobacter suboxydans* assay (6). *L. arabinosus* was found to be rather resistant to sulfathiazole. It required large amounts of the drug to bring about inhibition, and even at 10,000  $\gamma$  per 10 ml the inhibition was not complete. Low levels of sulfathiazole had more effect on the growth of *L. arabinosus* when PABA was absent than when it was in the medium. This is as expected, since more folic acid was synthesized when PABA was present. When 1  $\gamma$  of PABA was added per 10 ml of medium, 0.1 to 0.2  $\gamma$  of folic acid was found. On the other hand, when *L. arabinosus* was grown on medium containing no added PABA, no folic acid was detected in the medium as determined by *Streptococcus faecalis* assay.

Synergism was shown between the pterins and sulfathiazole with *Lactobacillus arabinosus* also. The diphenyl pterin (Compound 4) was the most active, since only 0.5  $\gamma$  of the compound per tube was needed to exhibit the synergistic effect without PABA in the medium, and 2  $\gamma$  with PABA. The 6,7-dicarboxy pterin (Compound 2) showed very slight if any effect, and the 7-carboxy derivative (Compound 3) was somewhat more effective, as was 2,4-diaminopyrimido(4,5-b)pyrazine (Compound 5). The dimethyl pterin (Compound 1) was intermediate in its action.

*Antagonism of Inhibition by Folic Acid*—The results showing the antagonism of pterin and sulfonamide inhibition of growth of *Escherichia coli* and *Staphylococcus aureus* by folic acid are presented in Table V. Folic acid completely overcame the inhibition of growth of these organisms, caused by low levels of pterin and sulfonamide. When higher levels of pterin and sulfathiazole were used, the antagonism was only partial. The antagonism seemed to be competitive regardless of whether the sulfathiazole or pterin was varied.

On the other hand, when the inhibition of pterin or sulfonamide was studied separately, the effect of added folic acid was non-competitive in nature, as is shown in Tables VI and VII. When folic acid was added at levels of 0.1 to 10,000  $\gamma$  per tube, the same amount of pterin brought about inhibition of growth of *Escherichia coli* and *Staphylococcus aureus*. Although the folic acid concentration varied over a 10,000- to 100,000-fold range, 1500 to 2000  $\gamma$  of the dimethyl pterin (Compound 1) inhibited the growth of both organisms.

In the previous paper (1) it was reported that folic acid antagonized the pterin inhibition of *Lactobacillus arabinosus* when no PABA was present in the medium. It has been found that if 1  $\gamma$  of PABA was added per tube 5000  $\gamma$  of added folic acid had no antagonistic effect on the inhibition.

Folic acid when supplied at high levels antagonized the inhibition of growth of *Escherichia coli* and *Staphylococcus aureus* caused by sulfon-

amides Lampen and Jones (2) reported that folic acid did not overcome the sulfonamide inhibition of *E coli* and *S aureus*. However, these

TABLE V

*Antagonism of Pterin and Sulfonamide Inhibition of Growth of Escherichia coli and Staphylococcus aureus by Folic Acid*

	Pterin added*	Sulfonamide added†	Folic acid added‡	Galvanometer readings§
	$\gamma$ per 10 ml	$\gamma$ per 10 ml	$\gamma$ per 10 ml	
<i>Escherichia coli</i>	0	0	0	36
	250	10	0	100
	250	10	10	69
	250	10	100	48
	250	10	1,000	36
	500	10	0	100
	500	10	10	93
	500	10	100	77
	500	10	1,000	43
	500	10	10,000	36
	500	5	0	95
	500	5	10	88
	500	5	100	52
	500	5	1,000	38
<i>Staphylococcus aureus</i>	0	0	0	15
	100	5	0	93
	100	5	10	96
	100	5	100	59
	100	5	1,000	30
	100	5	10,000	18
	250	5	0	95
	250	5	10	96
	250	5	100	95
	250	5	1,000	85
	250	5	10,000	84
	100	10	0	92
	100	10	10	96
	100	10	100	97
	100	10	1,000	50
	100	10	10,000	22

\* 2,4-Diamino 6,7 dimethylpyrimido(4,5 b)pyrazine

† Sulfathiazole

‡ Synthetic folic acid

§ A reading of 100 represents no growth

workers used low levels of folic acid. From the data presented here, it is evident that 1000  $\gamma$  of folic acid per 10 ml of medium will produce approximately normal growth of both organisms up to a certain concentration

TABLE VI

*Non-Antagonistic Effect of Synthetic Pterin and Folic Acid on Growth of Escherichia coli and Staphylococcus aureus*

	Pterin added*	Folic acid added,† $\gamma$ per 10 ml						
		0	0.1	1	10	100	1000	10,000
<i>Escherichia coli</i>	$\gamma$ per 10 ml							
	500	36†		36	38	38	39	37
	1000	66		65	65	65	68	65
	1500	83		77	81	79	76	81
<i>Staphylococcus aureus</i>	2000	88		90	89	91	88	84
	500	44	12	41	38	38	38	
	1000	71	72	71	72	71	68	
	1500	96	95	95	94	96	95	

\* 2,4-Diamino 6,7-dimethylpyrimido(4,5-b)pyrazine

† Synthetic folic acid

‡ Galvanometer readings, 100 represents no growth

TABLE VII

*Antagonism of Sulfonamide Inhibition of Growth of Escherichia coli, Staphylococcus aureus, and Lactobacillus arabinosus by Folic Acid*

	Sulfa thiazole added	Folic acid added,* $\gamma$ per 10 ml							
		0	0.1	1	10	100	1000	5000	10,000
<i>Escherichia coli</i>	$\gamma$ per 10 ml								
	0	38†			38	37	38		38
	20	52			46	41	35		36
	50	86			68	39	37		37
	100	94			86	58	36		37
	1,000	95			93	92	90		54
<i>Staphylococcus aureus</i>	10,000	95			93	92	91		80
	0	15				16	15	15	
	50	48				43	25	17	
	100	68				57	30	20	
	500	83				75	24	24	
	1,000	85				86	80	50	
<i>Lactobacillus arabinosus</i> , with PABA	10,000	90				88	90	87	
	0	5	5	5	5				
	100	48	53	16	6				
	1,000	56	63	12	5				
<i>Lactobacillus arabinosus</i> , without PABA	4,000	60	56	32	6				
	0	10	9	10	10				
	100	45	53	22	7				
	1,000	55	62	15	5				
	4,000	57	50	28	9				

\* Synthetic folic acid

† Galvanometer readings, 100 represents no growth

of sulfathiazole Above 100  $\gamma$  of sulfathiazole per tube for *E coli* and above 500  $\gamma$  for *S aureus*, the antagonism is partial or absent This appears to be an example of non-competitive antagonism

The results of a study of the antagonism of sulfonamide inhibition of *Lactobacillus arabinosus* by folic acid are presented in Table VII It is evident that this antagonism is non-competitive whether PABA is present in the medium or not As soon as the requirement of *L arabinosus* for folic acid was met, the amount of sulfathiazole present was of no importance

#### DISCUSSION

The results presented in this paper demonstrate that certain 2,4-diamino pterins show marked antibacterial action against *Escherichia coli* and *Staphylococcus aureus* and act synergistically with sulfathiazole to inhibit the growth of bacteria that synthesize folic acid Synergism between the pterins and sulfonamides is to be expected, since they are competing with two different parts of the folic acid molecule Beerstecher and Shive (7) have suggested that synergism usually occurs when two inhibitors prevent the functioning of two different enzymes in one series of enzymatic reactions The synergism shown by sulfonamide and pterin is one in which there are two inhibitors, one of which prevents the functioning of an enzyme active in the synthesis of folic acid, and the other of which appears to prevent the formation of an enzyme in which folic acid probably acts as the prosthetic group The inhibitory pterins may prevent the formation of the protein complex by competing with folic acid for the same position on the protein surface

Folic acid has been shown in a previous report (1) to antagonize the inhibition of growth of *Streptococcus faecalis*, *Lactobacillus casei*, and *Lactobacillus arabinosus* (without PABA) caused by the 2,4-diamino pterins Folic acid overcomes pterin inhibition of growth of *Escherichia coli* and *Staphylococcus aureus* at low levels of the compounds, but only in the presence of sulfonamide The antagonism seems to be competitive within a narrow range As the level of sulfonamide or pterin is raised, more folic acid is required to overcome the inhibition

When the pterin is added alone, the antagonism of folic acid is ineffective No matter how much folic acid is added, a constant amount of pterin will inhibit growth The explanation of this is uncertain, but it may be that the pterin is preferentially adsorbed on the protein surface, or that little of the folic acid can penetrate the cell structure under these conditions and be used

Sulfathiazole and folic acid exhibit non-competitive antagonism at relatively low levels of sulfonamide for *Escherichia coli*, *Staphylococcus aureus*, and *Lactobacillus arabinosus* Since sulfonamides interfere with

the synthesis of folic acid and not with its use, it is logical that this antagonism should be non-competitive in nature

The results obtained in these studies point to the possibility of using the inhibitory pterins in conjunction with sulfonamides in therapy, thereby decreasing the amount of sulfonamides required. The possible use of these pterins in other bacterial infections not affected by sulfonamides should not be overlooked, since these compounds are effective against those organisms that require folic acid, whether they need the vitamin preformed or synthesize it.

#### SUMMARY

Several synthetic pterins have been shown to possess marked antibacterial properties for *Escherichia coli* and *Staphylococcus aureus*. These compounds exhibit synergism with sulfathiazole in inhibiting the growth of organisms that synthesize folic acid. This inhibition is antagonized competitively by folic acid at low levels of the pterin and sulfonamide.

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## LETTERS TO THE EDITORS

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### SEPARATION OF ANTINVASIN I INTO TWO COMPONENTS

Sirs

The presence of an antihyaluronidase factor in normal serum has been established. Since hyaluronidase, which mediates spreading and invasion of bacteria and venoms, is inactivated in the course of this enzymatic reaction, the term antinvasin I has been introduced for the substance which inactivates hyaluronidase.<sup>1</sup>

In the course of isolation and purification of antinvasin I from serum, two individual fractions have now been obtained. Each of these fractions alone is practically inactive, but recombination of the two fractions results in the restoration of the original antinvasin I activity, as indicated by the rapid destruction of hyaluronidase.

The method of separation of antinvasin I into two components has been established for hog serum. Preliminary evidence indicates that with a somewhat modified procedure antinvasin I of human and rat serum likewise may be separated into two components and that the activity can be recovered by recombination of the two fractions.

That the reaction of antinvasin I derived from various species is not specific has been confirmed and extended here in experiments with the two individual components. Cross-reactions with heterologous serum fractions showed that antinvasin I activity was restored when Fraction I from human serum was combined with Fraction II from hog serum or vice versa.

For the separation of Component I, hog serum is cooled to  $-2^{\circ}$ , the pH is adjusted to 8.4, and cold ethanol is slowly added to a total concentration of 25 per cent. Component II under these conditions remains essentially in solution. The precipitate which contains Component I is separated by centrifugation and dissolved at pH 7.5 in a solution 0.02 M with respect to sodium chloride.

Component II is precipitated from the supernatant solution by readjusting the hydrogen ion concentration to 6.1. The precipitate containing Component II is collected by centrifugation, dissolved in water, and neutralized. The antinvasin I activity of whole hog serum, of the two individual fractions, and of the recombined components is demonstrated in the table. The viscosimetric method previously described was used for testing antinvasin I activity.<sup>1</sup>

<sup>1</sup> Haas, E., *J. Biol. Chem.*, **163**, 63 (1946)

Experiment No	Test substance	Antinvasin I activity
		<i>per cent</i>
I	Serum (0.1 ml)	100
II	Fraction I obtained from 0.1 ml serum	14
III	" II " " 0.1 " "	7
IV	Fractions I and II obtained from 0.1 ml serum, combined	100

The results, summarized in the table, indicate that for antinvasin I activity the participation of at least two components is required. The small activity observed in Experiments II and III presumably is due to incomplete separation of the two components. Both components are apparently of protein nature. The one present predominantly in Fraction II is heat-labile, incubation for 30 minutes at 60° results in a complete loss of activity. The other component, present essentially in Fraction I, is less heat-sensitive and retains its activity under the conditions indicated. Based on this difference in response to elevated temperature a second method of separation of the two components is provided.

Thus it has been shown that there are at least two components of antinvasin I and that they can be separated by fractionation with ethanol or by selective denaturation at elevated temperature.

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Received for publication, August 11, 1947



# THE ACTION OF RIBONUCLEINASE ON YEAST NUCLEIC ACID\*

Sirs

The use of enzymes as tools for the elucidation of the structure of ribonucleic acid which was introduced by Thannhauser<sup>1</sup> has been greatly stimulated by the isolation of ribonuclease in crystalline form by Kunitz<sup>2</sup>. The mechanism of the action of this enzyme is as yet unknown except for the fact that it involves the transformation of phosphoric acid diester groups into phosphoric acid monoester groups<sup>3</sup>.

We found that the hydrolysis of yeast ribonucleic acid by *N* sodium hydroxide as well as by ribonuclease is accompanied by the appearance of organic phosphorus compounds from which inorganic phosphate is rapidly formed during a subsequent short incubation with acid prostate phosphatase,<sup>4</sup> while ribonucleic acid itself is completely resistant against prostate phosphatase under the same conditions. The effect of alkali on nucleic acid differs sharply from that of ribonuclease, inasmuch as after treatment with *N* sodium hydroxide the P groups are quantitatively released as inorganic P during the subsequent incubation with prostate phosphatase, while an exhaustive hydrolysis with ribonuclease renders only approximately 25 per cent of the total P groups hydrolyzable by prostate phosphatase.

Since, under the conditions of our experiments, all mononucleotides are practically quantitatively dephosphorylated by prostate phosphatase, this means that alkali hydrolysis results in the complete cleavage of ribonucleic acids to mononucleotides, whereas after exhaustive digestion of ribonucleic acid with ribonuclease, at least 75 per cent of the P groups are present in the form of lower polynucleotides.

By the application of a new hydrolysis method<sup>5</sup> which permits the quantitative partition of ribonucleotide mixtures into purine and pyrimidine nucleotides, it could be demonstrated that the inorganic phosphate resulting

\* This study was aided by grants from the Rockefeller Foundation, the American Cancer Society, Inc., the Godfrey H. Hyams Trust Fund, the Bingham Associates Fund, and the Charlton Fund.

<sup>1</sup> Thannhauser, S. J., *Z. physiol. Chem.*, **91**, 329 (1914).

<sup>2</sup> Kunitz, M., *J. Gen. Physiol.*, **24**, 15 (1940).

<sup>3</sup> Allen, F. W., and Eiler, J. J., *J. Biol. Chem.*, **137**, 757 (1941).

<sup>4</sup> Bolomey and Allen (*J. Biol. Chem.*, **144**, 113 (1942)) have already reported that the dephosphorylation of ribonucleic acid by almond phosphatase is greatly increased when it is preceded by incubation of the substrate with ribonuclease.

<sup>5</sup> Schmidt, G., Cubiles, R., and Thannhauser, S. J., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **12** (1947).

from the combined action of ribonucleinase and prostate phosphatase on yeast ribonucleic acid originates exclusively from the pyrimidine nucleotide groups of ribonucleic acid. The acid-soluble polynucleotide fraction formed by the combined action of both enzymes was isolated in good yield. It was found to be resistant against prostate phosphatase. The ratio, purine nucleotide P to pyrimidine nucleotide P, in this polynucleotide fraction practically equals 2:1 as compared with the ratio 2:2 found in the original ribonucleic acid.

We conclude from these observations that the action of ribonucleinase involves specifically the pyrimidine nucleotide groups of ribonucleic acid. This conclusion is essentially in agreement with observations recently reported by Loring, Carpenter, and Roll.<sup>6</sup> In contrast to the opinion of these authors we conclude that not only the slowly diffusible but also a considerable portion of the fast diffusible split-products are polynucleotides.

The application of prostate phosphatase to the analysis of nucleotide mixtures is a suitable means for the quantitative partition of monoester and diester P groups. It is particularly valuable for the purpose of estimating the maximally possible amount of mononucleotides present in such mixtures. In the isolation of ribopolynucleotides, prostate phosphatase can be used with great advantage for the complete removal of mononucleotides.

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Received for publication, September 9, 1947

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<sup>6</sup> Loring, H. S., Carpenter, F. H., and Roll, P. M., *J. Biol. Chem.*, **169**, 601 (1947).

# THE ENZYMATIC MECHANISM OF ARGININE FORMATION FROM CITRULLINE\*

<sup>4</sup> *Sirs*

The synthesis of arginine from citrulline and glutamic or aspartic acid in kidney slices,<sup>1</sup> and from citrulline, glutamic acid, and catalytic amounts of ATP in kidney and liver homogenates,<sup>2</sup> has hitherto been effected only under aerobic conditions. A soluble enzyme, free of pyridine nucleotides, has now been obtained by alcohol fractionation of a buffered extract of beef

Micromoles substrate added					Micromoles found	
DL-Aspartic acid	L-Glutamic acid	Oxalacetic acid	L-Citrulline	L-Phosphoglyceric acid	Urea*	L-Malic acid
40			20	30	9.3†	9.5
	20		20	30	0.2	0.2
	20	30	20	30	8.6	9.0
		30	20	30	0.1	0.0
	20	30		30	0.0	0.2
40				30	0.0	0.2
40			20		0.9	

\* Since the preparation contained arginase, arginine was estimated as urea.

† When phosphoglyceric was replaced by 8 micromoles of ATP, 3.2 micromoles of urea were found, inhibition was observed with higher ATP concentrations. The reaction mixtures were incubated at 38° for 60 minutes. Each tube contained  $2.2 \times 10^{-3}$  M  $MgSO_4$ ,  $3.3 \times 10^{-4}$  M ATP,  $3.3 \times 10^{-2}$  M potassium phosphate, pH 7.4, expressed as final concentrations, 0.6 ml of thoroughly dialyzed enzyme solution containing 28 mg of protein, and neutralized substrates as indicated in the table. The final volume was 3 ml. Urea was estimated colorimetrically (Archibald, R. M., *J. Biol. Chem.*, **157**, 507 (1945)) and malic acid was estimated enzymatically (Ochoa, S., Mehler, A., and Kornberg, A., *J. Biol. Chem.*, **167**, 871 (1947), and personal communication).

liver acetone powder which catalyzes the anaerobic formation of arginine and malic acid from citrulline and aspartic acid in the presence of  $Mg^{++}$ , catalytic amounts of ATP, and phosphoglyceric acid as ATP generator (see the table). Aspartic acid may be replaced by a mixture of glutamic and oxalacetic acids because, under these conditions, aspartic acid is formed by transamination. The reactants may therefore be restricted to aspartic acid, citrulline, and high energy phosphate.

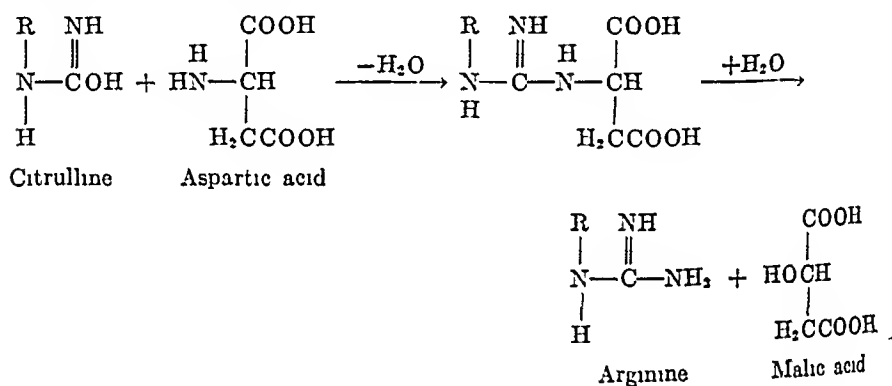
\* Aided by a grant from the United States Public Health Service.

<sup>1</sup> Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **141**, 717 (1941).

<sup>2</sup> Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **166**, 239 (1946), **166**, 251 (1946).

The failure of slices and homogenates to form arginine anaerobically can be ascribed to the fact that aerobic conditions are required for the generation of ATP by oxidation of keto acids<sup>3</sup>. Aerobically then glutamic acid must act as a substrate for ATP generation, as a precursor of oxalacetic acid, and in the formation of aspartic acid by transamination. It is of interest that in homogenates aerobic arginine production is accelerated by the addition of oxalacetic acid<sup>4</sup>.

The experimental data fail to support the hypothesis of transamination which postulates the oxidative removal of 2 H atoms<sup>1</sup>. The reaction may be formulated as an exchange of an OH and an NH<sub>2</sub> group in a manner analogous to transamination, it is assumed that citrulline is in equilibrium with an isourea form<sup>5</sup>.



In assigning a precise rôle to the high energy phosphate, one possibility is phosphorylation of the hydroxyl group in the isourea form of citrulline, in which case H<sub>3</sub>PO<sub>4</sub> rather than H<sub>2</sub>O would be split off on condensation.

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Received for publication, September 18, 1947

<sup>3</sup> Ochoa, S., *J. Biol. Chem.*, **155**, 87 (1944).

<sup>4</sup> Unpublished data of the author. See also Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **169**, 461 (1947).

<sup>5</sup> R =  $\begin{array}{cccc} \text{H} & \text{H} & \text{H} & \text{H} \\ -\text{C}- & \text{C}- & \text{C}- & \text{C}- \\ \text{H} & \text{H} & \text{H} & \text{NH}_2 \end{array} \text{COOH}$

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